# COMPLEMENTARY DNAs

## Related Applications

**[0001]** The present application is a divisional application of United States Patent Application Serial No. 09/247,155 filed on February 9, 1999, which claims priority from United States Provisional Patent Application Serial No. 60/074,121 filed February 9, 1998, United States Provisional Patent Application Serial No. 60/081,563, filed April 13, 1998, United States Provisional Patent Application Serial No. 60/096,116, filed August 10, 1998, and United States Provisional Patent Application Serial No. 60/099,273 filed September 4, 1998, the disclosures of which are incorporated herein by reference in their entirety.

**[0002]** Table I lists the SEQ ID Nos. of the extended cDNAs in the present application, the SEQ ID Nos. of the extended cDNAs in the provisional applications, and the identities of the provisional applications in which the extended cDNAs were disclosed.



## BACKGROUND OF THE INVENTION

**[0003]** The estimated 50,000-100,000 genes scattered along the human chromosomes offer tremendous promise for the understanding, diagnosis, and treatment of human diseases. In addition, probes capable of specifically hybridizing to loci distributed throughout the human genome find applications in the construction of high resolution chromosome maps and in the identification of individuals.

**[0004]** In the past, the characterization of even a single human gene was a painstaking process, requiring years of effort. Recent developments in the areas of cloning vectors, DNA sequencing, and computer technology have merged to greatly accelerate the rate at which human genes can be isolated, sequenced, mapped, and characterized. Cloning vectors such as yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs) are able to accept DNA inserts ranging from 300 to 1000 kilobases (kb) or 100-400 kb in length respectively, thereby facilitating the manipulation and ordering of DNA sequences distributed over great distances on the human chromosomes. Automated DNA sequencing machines permit the rapid sequencing of human genes.

Bioinformatics software enables the comparison of nucleic acid and protein sequences, thereby assisting in the characterization of human gene products.

**[0005]** Currently, two different approaches are being pursued for identifying and characterizing the genes distributed along the human genome. In one approach, large fragments of genomic DNA are isolated, cloned, and sequenced. Potential open reading frames in these genomic sequences are identified using bio-informatics software. However, this approach entails sequencing large stretches of human DNA which do not encode proteins in order to find the protein encoding sequences scattered throughout the genome. In addition to requiring extensive sequencing, the bio-informatics software may mischaracterize the genomic sequences obtained. Thus, the software may produce false positives in which non-coding DNA is mischaracterized as coding DNA or false negatives in which coding DNA is mislabeled as non-coding DNA.

**[0006]** An alternative approach takes a more direct route to identifying and characterizing human genes. In this approach, complementary DNAs (cDNAs) are synthesized from isolated messenger RNAs (mRNAs) which encode human proteins. Using this approach, sequencing is only performed on DNA which is derived from protein coding portions of the genome. Often, only short stretches of the cDNAs are sequenced to obtain sequences called expressed sequence tags (ESTs). The ESTs may then be used to isolate or purify extended cDNAs which include sequences adjacent to the EST sequences. The extended cDNAs may contain all of the sequence of the EST which was used to obtain them or only a portion of the sequence of the EST which was used to obtain them. In addition, the extended cDNAs may contain the full coding sequence of the gene from which the EST was derived or, alternatively, the extended cDNAs may include portions of the coding sequence of the gene from which the EST was derived. It will be appreciated that there may be several extended cDNAs which include the EST sequence as a result of alternate splicing or the activity of alternative promoters.

**[0007]** In the past, the short EST sequences which could be used to isolate or purify extended cDNAs were often obtained from oligo-dT primed cDNA libraries. Accordingly, they mainly corresponded to the 3′ untranslated region of the mRNA. In part, the prevalence of EST sequences derived from the 3′ end of the mRNA is a result of the fact that typical techniques for obtaining cDNAs, are not well suited for isolating

cDNA sequences derived from the 5′ ends of mRNAs. (Adams et al., *Nature* **377**:174, 1996, Hillier et al., *Genome Res.* **6**:807-828, 1996).

**[0008]** In addition, in those reported instances where longer cDNA sequences have been obtained, the reported sequences typically correspond to coding sequences and do not include the full 5′ untranslated region of the mRNA from which the cDNA is derived. Such incomplete sequences may not include the first exon of the mRNA, particularly in situations where the first exon is short. Furthermore, they may not include some exons, often short ones, which are located upstream of splicing sites. Thus, there is a need to obtain sequences derived from the 5′ ends of mRNAs which can be used to obtain extended cDNAs which may include the 5′ sequences contained in the 5′ ESTs.

**[0009]** While many sequences derived from human chromosomes have practical applications, approaches based on the identification and characterization of those chromosomal sequences which encode a protein product are particularly relevant to diagnostic and therapeutic uses. Of the 50,000-100,000 protein coding genes, those genes encoding proteins which are secreted from the cell in which they are synthesized, as well as the secreted proteins themselves, are particularly valuable as potential therapeutic agents. Such proteins are often involved in cell to cell communication and may be responsible for producing a clinically relevant response in their target cells.

**[0010]** In fact, several secretory proteins, including tissue plasminogen activator, G-CSF, GM-CSF, erythropoietin, human growth hormone, insulin, interferon-α, interferon-β, interferon-γ, and interleukin-2, are currently in clinical use. These proteins are used to treat a wide range of conditions, including acute myocardial infarction, acute ischemic stroke, anemia, diabetes, growth hormone deficiency, hepatitis, kidney carcinoma, chemotherapy induced neutropenia and multiple sclerosis. For these reasons, extended cDNAs encoding secreted proteins or portions thereof represent a particularly valuable source of therapeutic agents. Thus, there is a need for the identification and characterization of secreted proteins and the nucleic acids encoding them.

**[0011]** In addition to being therapeutically useful themselves, secretory proteins include short peptides, called signal peptides, at their amino termini which direct their secretion. These signal peptides are encoded by the signal sequences located at the 5′

ends of the coding sequences of genes encoding secreted proteins. Because these signal peptides will direct the extracellular secretion of any protein to which they are operably linked, the signal sequences may be exploited to direct the efficient secretion of any protein by operably linking the signal sequences to a gene encoding the protein for which secretion is desired. This may prove beneficial in gene therapy strategies in which it is desired to deliver a particular gene product to cells other than the cell in which it is produced. Signal sequences encoding signal peptides also find application in simplifying protein purification techniques. In such applications, the extracellular secretion of the desired protein greatly facilitates purification by reducing the number of undesired proteins from which the desired protein must be selected. Thus, there exists a need to identify and characterize the 5′ portions of the genes for secretory proteins which encode signal peptides.

**[0012]** Public information on the number of human genes for which the promoters and upstream regulatory regions have been identified and characterized is quite limited. In part, this may be due to the difficulty of isolating such regulatory sequences. Upstream regulatory sequences such as transcription factor binding sites are typically too short to be utilized as probes for isolating promoters from human genomic libraries. Recently, some approaches have been developed to isolate human promoters. One of them consists of making a CpG island library (Cross, S.H. et al., Purification of CpG Islands using a Methylated DNA Binding Column, Nature Genetics 6: 236-244 (1994)). The second consists of isolating human genomic DNA sequences containing SpeI binding sites by the use of SpeI binding protein. (Mortlock et al., *Genome Res.* **6**:327-335, 1996). Both of these approaches have their limits due to a lack of specificity or of comprehensiveness.

**[0013]** 5′ ESTs and extended cDNAs obtainable therefrom may be used to efficiently identify and isolate upstream regulatory regions which control the location, developmental stage, rate, and quantity of protein synthesis, as well as the stability of the mRNA. (Theil *et al.*, *BioFactors* **4**:87-93, (1993). Once identified and characterized, these regulatory regions may be utilized in gene therapy or protein purification schemes to obtain the desired amount and locations of protein synthesis or to inhibit, reduce, or prevent the synthesis of undesirable gene products.

**[0014]** In addition, ESTs containing the 5′ ends of secretory protein genes or extended cDNAs which include sequences adjacent to the sequences of the ESTs may include sequences useful as probes for chromosome mapping and the identification of individuals. Thus, there is a need to identify and characterize the sequences upstream of the 5′ coding sequences of genes encoding secretory proteins.

## SUMMARY OF THE INVENTION

**[0015]** The present invention relates to purified, isolated, or recombinant extended cDNAs which encode secreted proteins or fragments thereof. Preferably, the purified, isolated or recombinant cDNAs contain the entire open reading frame of their corresponding mRNAs, including a start codon and a stop codon. For example, the extended cDNAs may include nucleic acids encoding the signal peptide as well as the mature protein. Alternatively, the extended cDNAs may contain a fragment of the open reading frame. In some embodiments, the fragment may encode only the sequence of the mature protein. Alternatively, the fragment may encode only a portion of the mature protein. A further aspect of the present invention is a nucleic acid which encodes the signal peptide of a secreted protein.

**[0016]** The present extended cDNAs were obtained using ESTs which include sequences derived from the authentic 5′ ends of their corresponding mRNAs. As used herein the terms "EST" or "5′ EST" refer to the short cDNAs which were used to obtain the extended cDNAs of the present invention. As used herein, the term "extended cDNA" refers to the cDNAs which include sequences adjacent to the 5′ EST used to obtain them. The extended cDNAs may contain all or a portion of the sequence of the EST which was used to obtain them. The term "corresponding mRNA" refers to the mRNA which was the template for the cDNA synthesis which produced the 5′ EST. As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. Individual extended cDNA clones isolated from a cDNA library have been conventionally purified to electrophoretic homogeneity. The sequences obtained from these clones could not be obtained directly either from the library or from total human DNA. The extended cDNA clones are not naturally occurring as such, but rather

are obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). The conversion of mRNA into a cDNA library involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection. Thus, creating a cDNA library from messenger RNA and subsequently isolating individual clones from that library results in an approximately $10^4$-$10^6$ fold purification of the native message. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

**[0017]** As used herein, the term "isolated" requires that the material be removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide present in a living animal is not isolated, but the same polynucleotide, separated from some or all of the coexisting materials in the natural system, is isolated.

**[0018]** As used herein, the term "recombinant" means that the extended cDNA is adjacent to "backbone" nucleic acid to which it is not adjacent in its natural environment. Additionally, to be "enriched" the extended cDNAs will represent 5% or more of the number of nucleic acid inserts in a population of nucleic acid backbone molecules. Backbone molecules according to the present invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. Preferably, the enriched extended cDNAs represent 15% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. More preferably, the enriched extended cDNAs represent 50% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. In a highly preferred embodiment, the enriched extended cDNAs represent 90% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. "Stringent", "moderate," and "low" hybridization conditions are as defined in Example 29.

**[0019]** Unless otherwise indicated, a "complementary" sequence is fully complementary. Thus, extended cDNAs encoding secreted polypeptides or fragments thereof which are present in cDNA libraries in which one or more extended cDNAs encoding secreted polypeptides or fragments thereof make up 5% or more of the number

of nucleic acid inserts in the backbone molecules are "enriched recombinant extended cDNAs" as defined herein. Likewise, extended cDNAs encoding secreted polypeptides or fragments thereof which are in a population of plasmids in which one or more extended cDNAs of the present invention have been inserted such that they represent 5% or more of the number of inserts in the plasmid backbone are " enriched recombinant extended cDNAs" as defined herein. However, extended cDNAs encoding secreted polypeptides or fragments thereof which are in cDNA libraries in which the extended cDNAs encoding secreted polypeptides or fragments thereof constitute less than 5% of the number of nucleic acid inserts in the population of backbone molecules, such as libraries in which backbone molecules having a cDNA insert encoding a secreted polypeptide are extremely rare, are not "enriched recombinant extended cDNAs."

**[0020]** In particular, the present invention relates to extended cDNAs which were derived from genes encoding secreted proteins. As used herein, a "secreted" protein is one which, when expressed in a suitable host cell, is transported across or through a membrane, including transport as a result of signal peptides in its amino acid sequence. "Secreted" proteins include without limitation proteins secreted wholly (e.g. soluble proteins), or partially (e.g. receptors) from the cell in which they are expressed. "Secreted" proteins also include without limitation proteins which are transported across the membrane of the endoplasmic reticulum.

**[0021]** Extended cDNAs encoding secreted proteins may include nucleic acid sequences, called signal sequences, which encode signal peptides which direct the extracellular secretion of the proteins encoded by the extended cDNAs. Generally, the signal peptides are located at the amino termini of secreted proteins.

**[0022]** Secreted proteins are translated by ribosomes associated with the "rough" endoplasmic reticulum. Generally, secreted proteins are co-translationally transferred to the membrane of the endoplasmic reticulum. Association of the ribosome with the endoplasmic reticulum during translation of secreted proteins is mediated by the signal peptide. The signal peptide is typically cleaved following its co-translational entry into the endoplasmic reticulum. After delivery to the endoplasmic reticulum, secreted proteins may proceed through the Golgi apparatus. In the Golgi apparatus, the proteins

may undergo post-translational modification before entering secretory vesicles which transport them across the cell membrane.

**[0023]** The extended cDNAs of the present invention have several important applications. For example, they may be used to express the entire secreted protein which they encode. Alternatively, they may be used to express portions of the secreted protein. The portions may comprise the signal peptides encoded by the extended cDNAs or the mature proteins encoded by the extended cDNAs (i.e. the proteins generated when the signal peptide is cleaved off). The portions may also comprise polypeptides having at least 10 consecutive amino acids encoded by the extended cDNAs. Alternatively, the portions may comprise at least 15 consecutive amino acids encoded by the extended cDNAs. In some embodiments, the portions may comprise at least 25 consecutive amino acids encoded by the extended cDNAs. In other embodiments, the portions may comprise at least 40 amino acids encoded by the extended cDNAs.

**[0024]** Antibodies which specifically recognize the entire secreted proteins encoded by the extended cDNAs or fragments thereof having at least 10 consecutive amino acids, at least 15 consecutive amino acids, at least 25 consecutive amino acids, or at least 40 consecutive amino acids may also be obtained as described below. Antibodies which specifically recognize the mature protein generated when the signal peptide is cleaved may also be obtained as described below. Similarly, antibodies which specifically recognize the signal peptides encoded by the extended cDNAs may also be obtained.

**[0025]** In some embodiments, the extended cDNAs include the signal sequence. In other embodiments, the extended cDNAs may include the full coding sequence for the mature protein (i.e. the protein generated when the signal polypeptide is cleaved off). In addition, the extended cDNAs may include regulatory regions upstream of the translation start site or downstream of the stop codon which control the amount, location, or developmental stage of gene expression. As discussed above, secreted proteins are therapeutically important. Thus, the proteins expressed from the cDNAs may be useful in treating or controlling a variety of human conditions. The extended cDNAs may also be used to obtain the corresponding genomic DNA. The term "corresponding genomic DNA" refers to the genomic DNA which encodes mRNA which includes the

sequence of one of the strands of the extended cDNA in which thymidine residues in the sequence of the extended cDNA are replaced by uracil residues in the mRNA.

**[0026]** The extended cDNAs or genomic DNAs obtained therefrom may be used in forensic procedures to identify individuals or in diagnostic procedures to identify individuals having genetic diseases resulting from abnormal expression of the genes corresponding to the extended cDNAs. In addition, the present invention is useful for constructing a high resolution map of the human chromosomes.

**[0027]** The present invention also relates to secretion vectors capable of directing the secretion of a protein of interest. Such vectors may be used in gene therapy strategies in which it is desired to produce a gene product in one cell which is to be delivered to another location in the body. Secretion vectors may also facilitate the purification of desired proteins.

**[0028]** The present invention also relates to expression vectors capable of directing the expression of an inserted gene in a desired spatial or temporal manner or at a desired level. Such vectors may include sequences upstream of the extended cDNAs such as promoters or upstream regulatory sequences.

**[0029]** In addition, the present invention may also be used for gene therapy to control or treat genetic diseases. Signal peptides may also be fused to heterologous proteins to direct their extracellular secretion.

**[0030]** One embodiment of the present invention is a purified or isolated nucleic acid comprising the sequence of one of SEQ ID NOs: 40-84 and 130-154 or a sequence complementary thereto. In one aspect of this embodiment, the nucleic acid is recombinant.

**[0031]** Another embodiment of the present invention is a purified or isolated nucleic acid comprising at least 10 consecutive bases of the sequence of one of SEQ ID NOs: 40-84 and 130-154 or one of the sequences complementary thereto. In one aspect of this embodiment, the nucleic acid comprises at least 15, 25, 30, 40, 50, 75, or 100 consecutive bases of one of the sequences of SEQ ID NOs: 40-84 and 130-154 or one of the sequences complementary thereto. The nucleic acid may be a recombinant nucleic acid.

**[0032]** Another embodiment of the present invention is a purified or isolated nucleic acid of at least 15 bases capable of hybridizing under stringent conditions to the sequence of one of SEQ ID NOs: 40-84 and 130-154 or a sequence complementary to one of the sequences of SEQ ID NOs: 40-84 and 130-154. In one aspect of this embodiment, the nucleic acid is recombinant.

**[0033]** Another embodiment of the present invention is a purified or isolated nucleic acid comprising the full coding sequences of one of SEQ ID Nos: 40-84 and 130-154 wherein the full coding sequence optionally comprises the sequence encoding signal peptide as well as the sequence encoding mature protein. In a preferred embodiment, the isolated or purified nucleic acid comprises the full coding sequence of one of SEQ ID Nos. 40-59, 61-73, 75, 77-82, and 130-154 wherein the full coding sequence comprises the sequence encoding signal peptide and the sequence encoding mature protein. In one aspect of this embodiment, the nucleic acid is recombinant.

**[0034]** A further embodiment of the present invention is a purified or isolated nucleic acid comprising the nucleotides of one of SEQ ID NOs: 40-84 and 130-154 which encode a mature protein. In a preferred embodiment, the purified or isolated nucleic acid comprises the nucleotides of one of SEQ ID NOs: 40-59, 61-75, 77-82, and 130-154 which encode a mature protein. In one aspect of this embodiment, the nucleic acid is recombinant.

**[0035]** Yet another embodiment of the present invention is a purified or isolated nucleic acid comprising the nucleotides of one of SEQ ID NOs: 40-84 and 130-154 which encode the signal peptide. In a preferred embodiment, the purified or isolated nucleic acid comprises the nucleotides of SEQ ID NOs: 40-59, 61-73, 75-82, 84, and 130-154 which encode the signal peptide. In one aspect of this embodiment, the nucleic acid is recombinant.

**[0036]** Another embodiment of the present invention is a purified or isolated nucleic acid encoding a polypeptide having the sequence of one of the sequences of SEQ ID NOs: 85-129 and 155-179.

**[0037]** Another embodiment of the present invention is a purified or isolated nucleic acid encoding a polypeptide having the sequence of a mature protein

included in one of the sequences of SEQ ID NOs: 85-129 and 155-179. In a preferred embodiment, the purified or isolated nucleic acid encodes a polypeptide having the sequence of a mature protein included in one of the sequences of SEQ ID NOs: 85-104, 106-120, 122-127, and 155-179.

**[0038]** Another embodiment of the present invention is a purified or isolated nucleic acid encoding a polypeptide having the sequence of a signal peptide included in one of the sequences of SEQ ID NOs: 85-129 and 155-179. In a preferred embodiment, the purified or isolated nucleic acid encodes a polypeptide having the sequence of a signal peptide included in one of the sequences of SEQ ID NOs: 85-104, 106-118, 120-127, 129, and 155-179.

**[0039]** Yet another embodiment of the present invention is a purified or isolated protein comprising the sequence of one of SEQ ID NOs: 85-129 and 155- 179.

**[0040]** Another embodiment of the present invention is a purified or isolated polypeptide comprising at least 10 consecutive amino acids of one of the sequences of SEQ ID NOs: 85-129 and 155- 179. In one aspect of this embodiment, the purified or isolated polypeptide comprises at least 15, 20, 25, 35, 50, 75, 100, 150 or 200 consecutive amino acids of one of the sequences of SEQ ID NOs: 85-129 and 155- 179. In still another aspect, the purified or isolated polypeptide comprises at least 25 consecutive amino acids of one of the sequences of SEQ ID NOs: 85-129 and 155- 179.

**[0041]** Another embodiment of the present invention is an isolated or purified polypeptide comprising a signal peptide of one of the polypeptides of SEQ ID NOs: 85-129 and 155- 179. In a preferred embodiment, the isolated or purified polypeptide comprises a signal peptide of one of the polypeptides of SEQ ID NOs: 85-104, 106-118, 120-127, 129, and 155-179.

**[0042]** Yet another embodiment of the present invention is an isolated or purified polypeptide comprising a mature protein of one of the polypeptides of SEQ ID NOs: 85-129 and 155- 179. In a preferred embodiment, the isolated or purified polypeptide comprises a mature protein of one of the polypeptides of SEQ ID NOs: 85-104, 106-120, 122-127, and 155-179. In a preferred embodiment, the purified or isolated

nucleic acid encodes a polypeptide having the sequence of a mature protein included in one of the sequences of SEQ ID NOs: 85-104, 106-120, 122-127, and 155-179.

**[0043]** A further embodiment of the present invention is a method of making a protein comprising one of the sequences of SEQ ID NO: 85-129 and 155-179, comprising the steps of obtaining a cDNA comprising one of the sequences of sequence of SEQ ID NO: 40-84 and 130-154, inserting the cDNA in an expression vector such that the cDNA is operably linked to a promoter, and introducing the expression vector into a host cell whereby the host cell produces the protein encoded by said cDNA. In one aspect of this embodiment, the method further comprises the step of isolating the protein.

**[0044]** Another embodiment of the present invention is a protein obtainable by the method described in the preceding paragraph.

**[0045]** Another embodiment of the present invention is a method of making a protein comprising the amino acid sequence of the mature protein contained in one of the sequences of SEQ ID NOs: 85-104, 106-120, 122-127, and 155-179 comprising the steps of obtaining a cDNA comprising one of the nucleotides sequence of sequence of SEQ ID NOs: 40-59, 61-75, 77-82, and 130-154 which encode for the mature protein, inserting the cDNA in an expression vector such that the cDNA is operably linked to a promoter, and introducing the expression vector into a host cell whereby the host cell produces the mature protein encoded by the cDNA. In one aspect of this embodiment, the method further comprises the step of isolating the protein.

**[0046]** Another embodiment of the present invention is a mature protein obtainable by the method described in the preceding paragraph.

**[0047]** In a preferred embodiment, the above method comprises a method of making a protein comprising the amino acid sequence of the mature protein contained in one of the sequences of SEQ ID NOs. 85-104, 106-120, 122-127 and 155-179, comprising the steps of obtaining a cDNA comprising one of the nucleotide sequences of SEQ ID Nos. 40-59, 61-75, 77-82 and 130-154 which encode for the mature protein, inserting the cDNA in an expression vector such that the cDNA is operably linked to a promoter, and introducing the expression vector into a host cell

whereby the host cell produces the mature protein encoded by the cDNA. In one aspect of this embodiment, the method further comprises the step of isolating the protein.

**[0048]** Another embodiment of the present invention is a host cell containing the purified or isolated nucleic acids comprising the sequence of one of SEQ ID NOs: 40-84 and 130-154 or a sequence complementary thereto described herein.

**[0049]** Another embodiment of the present invention is a host cell containing the purified or isolated nucleic acids comprising the full coding sequences of one of SEQ ID NOs: 40-59, 61-73, 75, 77-82, and 130-154, wherein the full coding sequence comprises the sequence encoding signal peptide and the sequence encoding mature protein described herein.

**[0050]** Another embodiment of the present invention is a host cell containing the purified or isolated nucleic acids comprising the nucleotides of one of SEQ ID NOs: 40-84 and 130-154 which encode a mature protein which are described herein. Preferably, the host cell contains the purified or isolated nucleic acids comprising the nucleotides of one of SEQ ID NOs: 40-59, 61-75, 77-82, and 130-154 which encode a mature protein.

**[0051]** Another embodiment of the present invention is a host cell containing the purified or isolated nucleic acids comprising the nucleotides of one of SEQ ID NOs: 40-84 and 130-154 which encode the signal peptide which are described herein. Preferably, the host cell contains the purified or isolated nucleic acids comprising the nucleotides of one of SEQ ID Nos.: 40-59, 61-73, 75-82, 84, and 130-154 which encode the signal peptide.

**[0052]** Another embodiment of the present invention is a purified or isolated antibody capable of specifically binding to a protein having the sequence of one of SEQ ID NOs: 85-129 and 155-179. In one aspect of this embodiment, the antibody is capable of binding to a polypeptide comprising at least 10 consecutive amino acids of the sequence of one of SEQ ID NOs: 85-129 and 155-179.

**[0053]** Another embodiment of the present invention is an array of cDNAs or fragments thereof of at least 15 nucleotides in length which includes at least one of the sequences of SEQ ID NOs: 40-84 and 130-154, or one of the sequences complementary to

the sequences of SEQ ID NOs: 40-84 and 130-154, or a fragment thereof of at least 15 consecutive nucleotides. In one aspect of this embodiment, the array includes at least two of the sequences of SEQ ID NOs: 40-84 and 130-154, the sequences complementary to the sequences of SEQ ID NOs: 40-84 and 130-154, or fragments thereof of at least 15 consecutive nucleotides. In another aspect of this embodiment, the array includes at least five of the sequences of SEQ ID NOs: 40-84 and 130-154, the sequences complementary to the sequences of SEQ ID NOs: 40-84 and 130-154, or fragments thereof of at least 15 consecutive nucleotides.

**[0054]** A further embodiment of the invention encompasses purified polynucleotides comprising an insert from a clone deposited in a deposit having an accession number selected from the group consisting of the accession numbers listed in Table VI or a fragment thereof comprising a contiguous span of at least 8, 10, 12, 15, 20, 25, 40, 60, 100, or 200 nucleotides of said insert. An additional embodiment of the invention encompasses purified polypeptides which comprise, consist of, or consist essentially of an amino acid sequence encoded by the insert from a clone deposited in a deposit having an accession number selected from the group consisting of the accession numbers listed in Table VI, as well as polypeptides which comprise a fragment of said amino acid sequence consisting of a signal peptide, a mature protein, or a contiguous span of at least 5, 8, 10, 12, 15, 20, 25, 40, 60, 100, or 200 amino acids encoded by said insert.

**[0055]** An additional embodiment of the invention encompasses purified polypeptides which comprise a contiguous span of at least 5, 8, 10, 12, 15, 20, 25, 40, 60, 100, or 200 amino acids of SEQ ID NOs: 85-129 and 155-179, wherein said contiguous span comprises at least one of the amino acid positions which was not shown to be identical to a public sequence in any of Figures 10 to 12. Also encompassed by the invention are purified polynucleotides encoding said polypeptides.

**[0056]** Another embodiment of the present invention is a computer readable medium having stored thereon a sequence selected from the group consisting of a cDNA code of SEQID NOs. 40-84 and 130-154 and a polypeptide code of SEQ ID NOs. 85-129 and 155-179.

**[0057]** Another embodiment of the present invention is a computer system comprising a processor and a data storage device wherein the data storage device has

stored thereon a sequence selected from the group consisting of a cDNA code of SEQID NOs. 40-84 and 130-154 and a polypeptide code of SEQ ID NOs. 85-129 and 155-179. In some embodiments the computer system further comprises a sequence comparer and a data storage device having reference sequences stored thereon. For example, the sequence comparer may comprise a computer program which indicates polymorphisms. In other aspects of the computer system, the system further comprises an identifier which identifies features in said sequence.

**[0058]** Another embodiment of the present invention is a method for comparing a first sequence to a reference sequence wherein the first sequence is selected from the group consisting of a cDNA code of SEQID NOs. 40-84 and 130-154 and a polypeptide code of SEQ ID NOs. 85-129 and 155-179 comprising the steps of reading the first sequence and the reference sequence through use of a computer program which compares sequences and

determining differences between the first sequence and the reference sequence with the computer program. In some embodiments of the method, the step of determining differences between the first sequence and the reference sequence comprises identifying polymorphisms.

**[0059]** Another embodiment of the present invention is a method for identifying a feature in a sequence selected from the group consisting of a cDNA code of SEQID NOs. 40-84 and 130-154 and a polypeptide code of SEQ ID NOs. 85-129 and 155-179 comprising the steps of reading the sequence through the use of a computer program which identifies features in sequences and identifying features in the sequence with said computer program.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0060]** **Figure 1** is a summary of a procedure for obtaining cDNAs which have been selected to include the 5′ ends of the mRNAs from which they are derived.

**[0061]** **Figure 2** is an analysis of the 43 amino terminal amino acids of all human SwissProt proteins to determine the frequency of false positives and false negatives using the techniques for signal peptide identification described herein.

**[0062]** **Figure 3** shows the distribution of von Heijne scores for 5′ ESTs in each of the categories described herein and the probability that these 5′ ESTs encode a signal peptide.

**[0063]** **Figure 4** shows the distribution of 5′ ESTs in each category and the number of 5′ ESTs in each category having a given minimum von Heijne's score.

**[0064]** **Figure 5** shows the tissues from which the mRNAs corresponding to the 5′ ESTs in each of the categories described herein were obtained.

**[0065]** **Figure 6** illustrates a method for obtaining extended cDNAs.

**[0066]** **Figure 7** is a map of pED6dpc2.

**[0067]** **Figure 8** provides a schematic description of the promoters isolated and the way they are assembled with the corresponding 5′ tags.

**[0068]** **Figure 9** describes the transcription factor binding sites present in each of these promoters.

**[0069]** **Figure 10** is an alignment of the proteins of SEQ ID NOs: 120 and 180 wherein the signal peptide is in italics, the predicted transmembrane segment is underlined, the experimentally determined transmembrane segment is double-underlined, and the ATP1G/PLMN/MAT8 signature is in bold.

**[0070]** **Figure 11** is an alignment of the proteins of SEQ ID NOs: 121 and 181 wherein the predicted transmembrane segment is underlined.

**[0071]** **Figure 12** is an alignment of the proteins of SEQ ID NOs: 128 and 182 wherein the PPPY motif is in bold.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

### I. Obtaining 5′ ESTs

**[0072]** The present extended cDNAs were obtained using 5′ ESTs which were isolated as described below.

A. Chemical Methods for Obtaining mRNAs having Intact 5′ Ends

**[0073]** In order to obtain the 5′ ESTs used to obtain the extended cDNAs of the present invention, mRNAs having intact 5′ ends must be obtained. Currently, there are two approaches for obtaining such mRNAs. One of these approaches is a chemical modification method involving derivatization of the 5′ ends of the mRNAs and selection of the derivatized mRNAs. The 5′ ends of eucaryotic mRNAs possess a structure referred to as a "cap" which comprises a guanosine methylated at the 7 position. The cap is joined to the first transcribed base of the mRNA by a 5′, 5′-triphosphate bond. In some instances, the 5′ guanosine is methylated in both the 2 and 7 positions. Rarely, the 5′ guanosine is trimethylated at the 2, 7 and 7 positions. In the chemical method for obtaining mRNAs having intact 5′ ends, the 5′ cap is specifically derivatized and coupled to a reactive group on an immobilizing substrate. This specific derivatization is based on the fact that only the ribose linked to the methylated guanosine at the 5′ end of the mRNA and the ribose linked to the base at the 3′ terminus of the mRNA, possess 2′, 3′-cis diols. Optionally, where the 3′ terminal ribose has a 2′, 3′-cis diol, the 2′, 3′-cis diol at the 3′ end may be chemically modified, substituted, converted, or eliminated, leaving only the ribose linked to the methylated guanosine at the 5′ end of the mRNA with a 2′, 3′-cis diol. A variety of techniques are available for eliminating the 2′, 3′-cis diol on the 3′ terminal ribose. For example, controlled alkaline hydrolysis may be used to generate mRNA fragments in which the 3′ terminal ribose is a 3′-phosphate, 2′-phosphate or (2′, 3′)-cyclophosphate. Thereafter, the fragment which includes the original 3′ ribose may be eliminated from the mixture through chromatography on an oligo-dT column. Alternatively, a base which lacks the 2′, 3′-cis diol may be added to the 3′ end of the mRNA using an RNA ligase such

as T4 RNA ligase. Example 1 below describes a method for ligation of pCp to the 3′ end of messenger RNA.

## EXAMPLE 1

Ligation of the Nucleoside Diphosphate pCp to the 3′ End of Messenger RNA

**[0074]** 1 µg of RNA was incubated in a final reaction medium of 10 µl in the presence of 5 U of $T_4$ phage RNA ligase in the buffer provided by the manufacturer (Gibco - BRL), 40 U of the RNase inhibitor RNasin (Promega) and, 2 µl of $^{32}$pCp (Amersham #PB 10208).

**[0075]** The incubation was performed at 37°C for 2 hours or overnight at 7-8°C.

**[0076]** Following modification or elimination of the 2′, 3′-cis diol at the 3′ ribose, the 2′, 3′-cis diol present at the 5′ end of the mRNA may be oxidized using reagents such as $NaBH_4$, $NaBH_3CN$, or sodium periodate, thereby converting the 2′, 3′-cis diol to a dialdehyde. Example 2 describes the oxidation of the 2′, 3′-cis diol at the 5′ end of the mRNA with sodium periodate.

## EXAMPLE 2

Oxidation of 2′, 3′-cis diol at the 5′ End of the mRNA

**[0077]** 0.1 OD unit of either a capped oligoribonucleotide of 47 nucleotides (including the cap) or an uncapped oligoribonucleotide of 46 nucleotides were treated as follows. The oligoribonucleotides were produced by in vitro transcription using the transcription kit "AmpliScribe T7" (Epicentre Technologies). As indicated below, the DNA template for the RNA transcript contained a single cytosine. To synthesize the uncapped RNA, all four NTPs were included in the in vitro transcription reaction. To obtain the capped RNA, GTP was replaced by an analogue of the cap, m7G(5′)ppp(5′)G. This compound, recognized by polymerase, was incorporated into the 5′ end of the nascent transcript during the step of initiation of transcription but was not capable of incorporation

during the extension step. Consequently, the resulting RNA contained a cap at its 5′ end. The sequences of the oligoribonucleotides produced by the in vitro transcription reaction were:

+Cap:

5′m7GpppGCAUCCUACUCCCAUCCAAUUCCACCCUAACUCCUCC CAUCUCCAC-3′ (SEQ ID NO:1)

-Cap:

5′-pppGCAUCCUACUCCCAUCCAAUUCCACCCUAACUCCUCCCAUC UCCAC-3′ (SEQ ID NO:2)

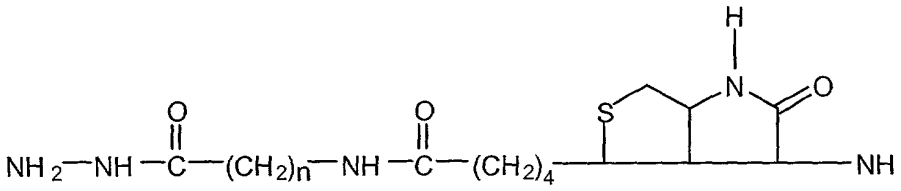
**[0078]** The oligoribonucleotides were dissolved in 9 µl of acetate buffer (0.1 M sodium acetate, pH 5.2) and 3 µl of freshly prepared 0.1 M sodium periodate solution. The mixture was incubated for 1 hour in the dark at 4°C or room temperature. Thereafter, the reaction was stopped by adding 4 µl of 10% ethylene glycol. The product was ethanol precipitated, resuspended in 10µl or more of water or appropriate buffer and dialyzed against water.

**[0079]** The resulting aldehyde groups may then be coupled to molecules having a reactive amine group, such as hydrazine, carbazide, thiocarbazide or semicarbazide groups, in order to facilitate enrichment of the 5′ ends of the mRNAs. Molecules having reactive amine groups which are suitable for use in selecting mRNAs having intact 5′ ends include avidin, proteins, antibodies, vitamins, ligands capable of specifically binding to receptor molecules, or oligonucleotides. Example 3 below describes the coupling of the resulting dialdehyde to biotin.

## EXAMPLE 3

Coupling of the Dialdehyde with Biotin

**[0080]** The oxidation product obtained in Example 2 was dissolved in 50 µl of sodium acetate at a pH of between 5 and 5.2 and 50 µl of freshly prepared 0.02 M solution of biotin hydrazide in a methoxyethanol/water mixture (1:1) of formula:




**[0081]** In the compound used in these experiments, n=5. However, it will be appreciated that other commercially available hydrazides may also be used, such as molecules of the formula above in which n varies from 0 to 5.

**[0082]** The mixture was then incubated for 2 hours at 37°C. Following the incubation, the mixture was precipitated with ethanol and dialyzed against distilled water.

**[0083]** Example 4 demonstrates the specificity of the biotinylation reaction.

## EXAMPLE 4

Specificity of Biotinylation

**[0084]** The specificity of the biotinylation for capped mRNAs was evaluated by gel electrophoresis of the following samples:

**[0085]** Sample 1. The 46 nucleotide uncapped in vitro transcript prepared as in Example 2 and labeled with $^{32}$pCp as described in Example 1.

**[0086]** Sample 2. The 46 nucleotide uncapped in vitro transcript prepared as in Example 2, labeled with $^{32}$pCp as described in Example 1, treated with the oxidation reaction of Example 2, and subjected to the biotinylation conditions of Example 3.

**[0087]** Sample 3. The 47 nucleotide capped in vitro transcript prepared as in Example 2 and labeled with $^{32}$pCp as described in Example 1.

**[0088]** Sample 4. The 47 nucleotide capped in vitro transcript prepared as in Example 2, labeled with $^{32}$pCp as described in Example 1, treated with the oxidation reaction of Example 2, and subjected to the biotinylation conditions of Example 3.

**[0089]** Samples 1 and 2 had identical migration rates, demonstrating that the uncapped RNAs were not oxidized and biotinylated. Sample 3 migrated more slowly than Samples 1 and 2, while Sample 4 exhibited the slowest migration. The difference in migration of the RNAs in Samples 3 and 4 demonstrates that the capped RNAs were specifically biotinylated.

**[0090]** In some cases, mRNAs having intact 5′ ends may be enriched by binding the molecule containing a reactive amine group to a suitable solid phase substrate such as the inside of the vessel containing the mRNAs, magnetic beads, chromatography matrices, or nylon or nitrocellulose membranes. For example, where the molecule having a reactive amine group is biotin, the solid phase substrate may be coupled to avidin or streptavidin. Alternatively, where the molecule having the reactive amine group is an antibody or receptor ligand, the solid phase substrate may be coupled to the cognate antigen or receptor. Finally, where the molecule having a reactive amine group comprises an oligonucleotide, the solid phase substrate may comprise a complementary oligonucleotide.

**[0091]** The mRNAs having intact 5′ ends may be released from the solid phase following the enrichment procedure. For example, where the dialdehyde is coupled to biotin hydrazide and the solid phase comprises streptavidin, the mRNAs may be released from the solid phase by simply heating to 95 degrees Celsius in 2% SDS. In some methods, the molecule having a reactive amine group may also be cleaved from the mRNAs having intact 5′ ends following enrichment. Example 5 describes the capture of

biotinylated mRNAs with streptavidin coated beads and the release of the biotinylated mRNAs from the beads following enrichment.

## EXAMPLE 5

Capture and Release of Biotinylated mRNAs Using Strepatividin Coated Beads

**[0092]** The streptavidin-coated magnetic beads were prepared according to the manufacturer's instructions (CPG Inc., USA). The biotinylated mRNAs were added to a hybridization buffer (1.5 M NaCl, pH 5 - 6). After incubating for 30 minutes, the unbound and nonbiotinylated material was removed. The beads were washed several times in water with 1% SDS. The beads obtained were incubated for 15 minutes at 95°C in water containing 2% SDS.

**[0093]** Example 6 demonstrates the efficiency with which biotinylated mRNAs were recovered from the streptavidin coated beads.

## EXAMPLE 6

Efficiency of Recovery of Biotinylated mRNAs

**[0094]** The efficiency of the recovery procedure was evaluated as follows. RNAs were labeled with $^{32}$pCp, oxidized, biotinylated and bound to streptavidin coated beads as described above. Subsequently, the bound RNAs were incubated for 5, 15 or 30 minutes at 95°C in the presence of 2% SDS.

**[0095]** The products of the reaction were analyzed by electrophoresis on 12% polyacrylamide gels under denaturing conditions (7 M urea). The gels were subjected to autoradiography. During this manipulation, the hydrazone bonds were not reduced.

**[0096]** Increasing amounts of nucleic acids were recovered as incubation times in 2% SDS increased, demonstrating that biotinylated mRNAs were efficiently recovered.

**[0097]** In an alternative method for obtaining mRNAs having intact 5′ ends, an oligonucleotide which has been derivatized to contain a reactive amine group is

specifically coupled to mRNAs having an intact cap. Preferably, the 3′ end of the mRNA is blocked prior to the step in which the aldehyde groups are joined to the derivatized oligonucleotide, as described above, so as to prevent the derivatized oligonucleotide from being joined to the 3′ end of the mRNA. For example, pCp may be attached to the 3′ end of the mRNA using T4 RNA ligase. However, as discussed above, blocking the 3′ end of the mRNA is an optional step. Derivatized oligonucleotides may be prepared as described below in Example 7.

## EXAMPLE 7

Derivatization of the Oligonucleotide

**[0098]** An oligonucleotide phosphorylated at its 3′ end was converted to a 3′ hydrazide in 3′ by treatment with an aqueous solution of hydrazine or of dihydrazide of the formula $H_2N(R1)NH_2$ at about 1 to 3 M, and at pH 4.5, in the presence of a carbodiimide type agent soluble in water such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at a final concentration of 0.3 M at a temperature of 8°C overnight.

**[0099]** The derivatized oligonucleotide was then separated from the other agents and products using a standard technique for isolating oligonucleotides.

**[0100]** As discussed above, the mRNAs to be enriched may be treated to eliminate the 3′ OH groups which may be present thereon. This may be accomplished by enzymatic ligation of sequences lacking a 3′ OH, such as pCp, as described above in Example 1. Alternatively, the 3′ OH groups may be eliminated by alkaline hydrolysis as described in Example 8 below.

## EXAMPLE 8

Alkaline Hydrolysis of mRNA

**[0101]** The mRNAs may be treated with alkaline hydrolysis as follows. In a total volume of 100µl of 0.1N sodium hydroxide, 1.5µg mRNA is incubated for 40 to 60 minutes at 4°C. The solution is neutralized with acetic acid and precipitated with ethanol.

**[0102]** Following the optional elimination of the 3′ OH groups, the diol groups at the 5′ ends of the mRNAs are oxidized as described below in Example 9.

## EXAMPLE 9

Oxidation of Diols

**[0103]** Up to 1 OD unit of RNA was dissolved in 9 µl of buffer (0.1 M sodium acetate, pH 6-7 or water) and 3 µl of freshly prepared 0.1 M sodium periodate solution. The reaction was incubated for 1 h in the dark at 4°C or room temperature. Following the incubation, the reaction was stopped by adding 4 µl of 10% ethylene glycol. Thereafter the mixture was incubated at room temperature for 15 minutes. After ethanol precipitation, the product was resuspended in 10µl or more of water or appropriate buffer and dialyzed against water.

**[0104]** Following oxidation of the diol groups at the 5′ ends of the mRNAs, the derivatized oligonucleotide was joined to the resulting aldehydes as described in Example 10.

## EXAMPLE 10

Reaction of Aldehydes with Derivatized Oligonucleotides

**[0105]** The oxidized mRNA was dissolved in an acidic medium such as 50 µl of sodium acetate pH 4-6. 50 µl of a solution of the derivatized oligonucleotide was added such that an mRNA:derivatized oligonucleotide ratio of 1:20 was obtained and mixture was reduced with a borohydride. The mixture was allowed to incubate for 2 h at 37°C or overnight (14 h) at 10°C. The mixture was ethanol precipitated, resuspended in

10μl or more of water or appropriate buffer and dialyzed against distilled water. If desired, the resulting product may be analyzed using acrylamide gel electrophoresis, HPLC analysis, or other conventional techniques.

**[0106]** Following the attachment of the derivatized oligonucleotide to the mRNAs, a reverse transcription reaction may be performed as described in Example 11 below.

## EXAMPLE 11

Reverse Transcription of mRNAs

**[0107]** An oligodeoxyribonucleotide was derivatized as follows. 3 OD units of an oligodeoxyribonucleotide of sequence ATCAAGAATTCGCACGAGACCATTA (SEQ ID NO:3) having 5'-OH and 3'-P ends were dissolved in 70 μl of a 1.5 M hydroxybenzotriazole solution, pH 5.3, prepared in dimethylformamide/water (75:25) containing 2 μg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The mixture was incubated for 2 h 30 min at 22°C. The mixture was then precipitated twice in $LiClO_4$/acetone. The pellet was resuspended in 200 μl of 0.25 M hydrazine and incubated at 8°C from 3 to 14 h. Following the hydrazine reaction, the mixture was precipitated twice in $LiClO_4$/acetone.

**[0108]** The messenger RNAs to be reverse transcribed were extracted from blocks of placenta having sides of 2 cm which had been stored at -80°C. The mRNA was extracted using conventional acidic phenol techniques. Oligo-dT chromatography was used to purify the mRNAs. The integrity of the mRNAs was checked by Northern-blotting.

**[0109]** The diol groups on 7 μg of the placental mRNAs were oxidized as described above in Example 9. The derivatized oligonucleotide was joined to the mRNAs as described in Example 10 above except that the precipitation step was replaced by an exclusion chromatography step to remove derivatized oligodeoxyribonucleotides which were not joined to mRNAs. Exclusion chromatography was performed as follows:

**[0110]** 10 ml of AcA34 (BioSepra#230151) gel were equilibrated in 50 ml of a solution of 10 mM Tris pH 8.0, 300 mM NaCl, 1 mM EDTA, and 0.05% SDS. The

mixture was allowed to sediment. The supernatant was eliminated and the gel was resuspended in 50 ml of buffer. This procedure was repeated 2 or 3 times.

**[0111]** A glass bead (diameter 3 mm) was introduced into a 2 ml disposable pipette (length 25 cm). The pipette was filled with the gel suspension until the height of the gel stabilized at 1 cm from the top of the pipette. The column was then equilibrated with 20 ml of equilibration buffer (10 mM Tris HCl pH 7.4, 20 mM NaCl).

**[0112]** 10 µl of the mRNA which had been reacted with the derivatized oligonucleotide were mixed in 39 µl of 10 mM urea and 2 µl of blue-glycerol buffer, which had been prepared by dissolving 5 mg of bromophenol blue in 60% glycerol (v/v), and passing the mixture through a filter with a filter of diameter 0.45 µm.

**[0113]** The column was loaded. As soon as the sample had penetrated, equilibration buffer was added. 100 µl fractions were collected. Derivatized oligonucleotide which had not been attached to mRNA appeared in fraction 16 and later fractions. Fractions 3 to 15 were combined and precipitated with ethanol.

**[0114]** The mRNAs which had been reacted with the derivatized oligonucleotide were spotted on a nylon membrane and hybridized to a radioactive probe using conventional techniques. The radioactive probe used in these hybridizations was an oligodeoxyribonucleotide of sequence TAATGGTCTCGTGCGAATTCTTGAT (SEQ ID NO:4) which was anticomplementary to the derivatized oligonucleotide and was labeled at its 5′ end with $^{32}P$. 1/10th of the mRNAs which had been reacted with the derivatized oligonucleotide was spotted in two spots on the membrane and the membrane was visualized by autoradiography after hybridization of the probe. A signal was observed, indicating that the derivatized oligonucleotide had been joined to the mRNA.

**[0115]** The remaining 9/10 of the mRNAs which had been reacted with the derivatized oligonucleotide was reverse transcribed as follows. A reverse transcription reaction was carried out with reverse transcriptase following the manufacturer's instructions. To prime the reaction, 50 pmol of nonamers with random sequence were used.

**[0116]** A portion of the resulting cDNA was spotted on a positively charged nylon membrane using conventional methods. The cDNAs were spotted on the

membrane after the cDNA:RNA heteroduplexes had been subjected to an alkaline hydrolysis in order to eliminate the RNAs. An oligonucleotide having a sequence identical to that of the derivatized oligonucleotide was labeled at its 5′ end with $^{32}P$ and hybridized to the cDNA blots using conventional techniques. Single-stranded cDNAs resulting from the reverse transcription reaction were spotted on the membrane. As controls, the blot contained 1 pmol, 100 fmol, 50 fmol, 10 fmol and 1 fmol respectively of a control oligodeoxyribonucleotide of sequence identical to that of the derivatized oligonucleotide. The signal observed in the spots containing the cDNA indicated that approximately 15 fmol of the derivatized oligonucleotide had been reverse transcribed.

**[0117]** These results demonstrate that the reverse transcription can be performed through the cap and, in particular, that reverse transcriptase crosses the 5′-P-P-P-5′ bond of the cap of eukaryotic messenger RNAs.

**[0118]** The single stranded cDNAs obtained after the above first strand synthesis were used as template for PCR reactions. Two types of reactions were carried out. First, specific amplification of the mRNAs for the alpha globin, dehydrogenase, pp15 and elongation factor E4 were carried out using the following pairs of oligodeoxyribonucleotide primers.

alpha-globin

GLO-S: CCG ACA AGA CCA ACG TCA AGG CCG C (SEQ ID NO:5)

GLO-As: TCA CCA GCA GGC AGT GGC TTA GGA G 3′ (SEQ ID NO:6)

dehydrogenase

3 DH-S: AGT GAT TCC TGC TAC TTT GGA TGG C (SEQ ID NO:7)

3 DH-As: GCT TGG TCT TGT TCT GGA GTT TAG A (SEQ ID NO:8)

pp15

PP15-S: TCC AGA ATG GGA GAC AAG CCA ATT T (SEQ ID NO:9)

PP15-As: AGG GAG GAG GAA ACA GCG TGA GTC C (SEQ ID NO:10)

Elongation factor E4

EFA1-S: ATG GGA AAG GAA AAG ACT CAT ATC A (SEQ ID NO:11)

EF1A-As: AGC AGC AAC AAT CAG GAC AGC ACA G (SEQ ID NO:12)

**[0119]** Non specific amplifications were also carried out with the antisense (_As) oligodeoxyribonucleotides of the pairs described above and a primer chosen from the sequence of the derivatized oligodeoxyribonucleotide (ATCAAGAATTCGCACGAGACCATTA) (SEQ ID NO:13).

**[0120]** A 1.5% agarose gel containing the following samples corresponding to the PCR products of reverse transcription was stained with ethidium bromide. (1/20th of the products of reverse transcription were used for each PCR reaction).

**[0121]** Sample 1: The products of a PCR reaction using the globin primers of SEQ ID NOs 5 and 6 in the presence of cDNA.

**[0122]** Sample 2: The products of a PCR reaction using the globin primers of SEQ ID NOs 5 and 6 in the absence of added cDNA.

**[0123]** Sample 3: The products of a PCR reaction using the dehydrogenase primers of SEQ ID NOs 7 and 8 in the presence of cDNA.

**[0124]** Sample 4: The products of a PCR reaction using the dehydrogenase primers of SEQ ID NOs 7 and 8 in the absence of added cDNA.

**[0125]** Sample 5: The products of a PCR reaction using the pp15 primers of SEQ ID NOs 9 and 10 in the presence of cDNA.

**[0126]** Sample 6: The products of a PCR reaction using the pp15 primers of SEQ ID NOs 9 and 10 in the absence of added cDNA.

**[0127]** Sample 7: The products of a PCR reaction using the EIE4 primers of SEQ ID NOs 11 and 12 in the presence of added cDNA.

**[0128]** Sample 8: The products of a PCR reaction using the EIE4 primers of SEQ ID NOs 11 and 12 in the absence of added cDNA.

**[0129]** In Samples 1, 3, 5 and 7, a band of the size expected for the PCR product was observed, indicating the presence of the corresponding sequence in the cDNA population.

**[0130]** PCR reactions were also carried out with the antisense oligonucleotides of the globin and dehydrogenase primers (SEQ ID NOs 6 and 8) and an oligonucleotide whose sequence corresponds to that of the derivatized oligonucleotide. The presence of PCR products of the expected size in the samples corresponding to samples 1 and 3 above indicated that the derivatized oligonucleotide had been incorporated.

**[0131]** The above examples summarize the chemical procedure for enriching mRNAs for those having intact 5′ ends. Further detail regarding the chemical approaches for obtaining mRNAs having intact 5′ ends are disclosed in International Application No. WO96/34981, published November 7, 1996, which is incorporated herein by reference. Strategies based on the above chemical modifications to the 5′ cap structure may be utilized to generate cDNAs which have been selected to include the 5′ ends of the mRNAs from which they are derived. In one version of such procedures, the 5′ ends of the mRNAs are modified as described above. Thereafter, a reverse transcription reaction is conducted to extend a primer complementary to the mRNA to the 5′ end of the mRNA. Single stranded RNAs are eliminated to obtain a population of cDNA/mRNA heteroduplexes in which the mRNA includes an intact 5′ end. The resulting heteroduplexes may be captured on a solid phase coated with a molecule capable of interacting with the molecule used to derivatize the 5′ end of the mRNA. Thereafter, the strands of the heteroduplexes are separated to recover single stranded first cDNA strands which include the 5′ end of the mRNA. Second strand cDNA synthesis may then proceed using conventional techniques. For example, the procedures disclosed in WO 96/34981 or in Carninci, P. et al. High-Efficiency Full-Length cDNA Cloning by Biotinylated CAP Trapper. **Genomics** 37:327-336 (1996), the disclosures of which are incorporated herein by reference, may be employed to select cDNAs which include the sequence derived from the 5′ end of the coding sequence of the mRNA.

**[0132]** Following ligation of the oligonucleotide tag to the 5′ cap of the mRNA, a reverse transcription reaction is conducted to extend a primer complementary to the mRNA to the 5′ end of the mRNA. Following elimination of the RNA component of

the resulting heteroduplex using standard techniques, second strand cDNA synthesis is conducted with a primer complementary to the oligonucleotide tag.

**[0133]** Figure 1 summarizes the above procedures for obtaining cDNAs which have been selected to include the 5′ ends of the mRNAs from which they are derived.

B. Enzymatic Methods for Obtaining mRNAs having Intact 5′ Ends

**[0134]** Other techniques for selecting cDNAs extending to the 5′ end of the mRNA from which they are derived are fully enzymatic. Some versions of these techniques are disclosed in Dumas Milne Edwards J.B. (Doctoral Thesis of Paris VI University, Le clonage des ADNc complets: difficultes et perspectives nouvelles. Apports pour l'etude de la regulation de l'expression de la tryptophane hydroxylase de rat, 20 Dec. 1993), EP0 625572 and Kato et al. Construction of a Human Full-Length cDNA Bank. Gene 150:243-250 (1994), the disclosures of which are incorporated herein by reference.

**[0135]** Briefly, in such approaches, isolated mRNA is treated with alkaline phosphatase to remove the phosphate groups present on the 5′ ends of uncapped incomplete mRNAs. Following this procedure, the cap present on full length mRNAs is enzymatically removed with a decapping enzyme such as T4 polynucleotide kinase or tobacco acid pyrophosphatase. An oligonucleotide, which may be either a DNA oligonucleotide or a DNA-RNA hybrid oligonucleotide having RNA at its 3′ end, is then ligated to the phosphate present at the 5′ end of the decapped mRNA using T4 RNA ligase. The oligonucleotide may include a restriction site to facilitate cloning of the cDNAs following their synthesis. Example 12 below describes one enzymatic method based on the doctoral thesis of Dumas.

**EXAMPLE 12**

Enzymatic Approach for Obtaining 5′ ESTs

**[0136]** Twenty micrograms of PolyA+ RNA were dephosphorylated using Calf Intestinal Phosphatase (Biolabs). After a phenol chloroform extraction, the cap

structure of mRNA was hydrolyzed using the Tobacco Acid Pyrophosphatase (purified as described by Shinshi et al., Biochemistry 15: 2185-2190, 1976) and a hemi 5'DNA/RNA-3' oligonucleotide having an unphosphorylated 5' end, a stretch of adenosine ribophosphate at the 3' end, and an EcoRI site near the 5' end was ligated to the 5'P ends of mRNA using the T4 RNA ligase (Biolabs). Oligonucleotides suitable for use in this procedure are preferably 30-50 bases in length. Oligonucleotides having an unphosphorylated 5' end may be synthesized by adding a fluorochrome at the 5' end. The inclusion of a stretch of adenosine ribophosphates at the 3' end of the oligonucleotide increases ligation efficiency. It will be appreciated that the oligonucleotide may contain cloning sites other than EcoRI.

**[0137]** Following ligation of the oligonucleotide to the phosphate present at the 5' end of the decapped mRNA, first and second strand cDNA synthesis may be carried out using conventional methods or those specified in EP0 625,572 and Kato et al. Construction of a Human Full-Length cDNA Bank. **Gene** 150:243-250 (1994), and Dumas Milne Edwards, *supra*, the disclosures of which are incorporated herein by reference. The resulting cDNA may then be ligated into vectors such as those disclosed in Kato et al. Construction of a Human Full-Length cDNA Bank. Gene 150:243-250 (1994) or other nucleic acid vectors known to those skilled in the art using techniques such as those described in Sambrook et al., Molecular Cloning: A Laboratory Manual 2d Ed., Cold Spring Harbor Laboratory Press, 1989, the disclosure of which is incorporated herein by reference.



## II. Characterization of 5' ESTs

**[0138]** The above chemical and enzymatic approaches for enriching mRNAs having intact 5' ends were employed to obtain 5' ESTs. First, mRNAs were prepared as described in Example 13 below.

## EXAMPLE 13

Preparation of mRNA

**[0139]** Total human RNAs or PolyA+ RNAs derived from 29 different tissues were respectively purchased from LABIMO and CLONTECH and used to generate 44 cDNA libraries as described below. The purchased RNA had been isolated from cells or tissues using acid guanidium thiocyanate-phenol-chloroform extraction (Chomczyniski, P and Sacchi, N., **Analytical Biochemistry** 162:156-159, 1987). PolyA+ RNA was isolated from total RNA (LABIMO) by two passes of oligodT chromatography, as described by Aviv and Leder (Aviv, H. and Leder, P., **Proc. Natl. Acad. Sci. USA** 69:1408-1412, 1972) in order to eliminate ribosomal RNA.

**[0140]** The quality and the integrity of the poly A+ were checked. Northern blots hybridized with a globin probe were used to confirm that the mRNAs were not degraded. Contamination of the PolyA+ mRNAs by ribosomal sequences was checked using RNAs blots and a probe derived from the sequence of the 28S RNA. Preparations of mRNAs with less than 5% of ribosomal RNAs were used in library construction. To avoid constructing libraries with RNAs contaminated by exogenous sequences (prokaryotic or fungal), the presence of bacterial 16S ribosomal sequences or of two highly expressed mRNAs was examined using PCR.

**[0141]** Following preparation of the mRNAs, the above described chemical and/or the enzymatic procedures for enriching mRNAs having intact 5′ ends discussed above were employed to obtain 5′ ESTs from various tissues. In both approaches an oligonucleotide tag was attached to the cap at the 5′ ends of the mRNAs. The oligonucleotide tag had an EcoRI site therein to facilitate later cloning procedures.

**[0142]** Following attachment of the oligonucleotide tag to the mRNA by either the chemical or enzymatic methods, the integrity of the mRNA was examined by performing a Northern blot with 200-500ng of mRNA using a probe complementary to the oligonucleotide tag.

## EXAMPLE 14

cDNA Synthesis Using mRNA Templates Having Intact 5′ Ends

**[0143]** For the mRNAs joined to oligonucleotide tags using both the chemical and enzymatic methods, first strand cDNA synthesis was performed using reverse transcriptase with random nonamers as primers. In order to protect internal EcoRI sites in the cDNA from digestion at later steps in the procedure, methylated dCTP was used for first strand synthesis. After removal of RNA by an alkaline hydrolysis, the first strand of cDNA was precipitated using isopropanol in order to eliminate residual primers.

**[0144]** For both the chemical and the enzymatic methods, the second strand of the cDNA was synthesized with a Klenow fragment using a primer corresponding to the 5'end of the ligated oligonucleotide described in Example 12. Preferably, the primer is 20-25 bases in length. Methylated dCTP was also used for second strand synthesis in order to protect internal EcoRI sites in the cDNA from digestion during the cloning process.

**[0145]** Following cDNA synthesis, the cDNAs were cloned into pBlueScript as described in Example 15 below.

## EXAMPLE 15

Insertion of cDNAs into BlueScript

**[0146]** Following second strand synthesis, the ends of the cDNA were blunted with T4 DNA polymerase (Biolabs) and the cDNA was digested with EcoRI. Since methylated dCTP was used during cDNA synthesis, the EcoRI site present in the tag was the only site which was hemi-methylated. Consequently, only the EcoRI site in the oligonucleotide tag was susceptible to EcoRI digestion. The cDNA was then size fractionated using exclusion chromatography (AcA, Biosepra). Fractions corresponding to cDNAs of more than 150 bp were pooled and ethanol precipitated. The cDNA was directionally cloned into the SmaI and EcoRI ends of the phagemid pBlueScript vector (Stratagene). The ligation mixture was electroporated into bacteria and propagated under appropriate antibiotic selection.

**[0147]** Clones containing the oligonucleotide tag attached were selected as described in Example 16 below.

## EXAMPLE 16

Selection of Clones Having the Oligonucleotide Tag Attached Thereto

**[0148]** The plasmid DNAs containing 5′ EST libraries made as described above were purified (Qiagen). A positive selection of the tagged clones was performed as follows. Briefly, in this selection procedure, the plasmid DNA was converted to single stranded DNA using gene II endonuclease of the phage F1 in combination with an exonuclease (Chang et al., **Gene 127**:95-8, 1993) such as exonuclease III or T7 gene 6 exonuclease. The resulting single stranded DNA was then purified using paramagnetic beads as described by Fry et al., **Biotechniques, 13**: 124-131, 1992. In this procedure, the single stranded DNA was hybridized with a biotinylated oligonucleotide having a sequence corresponding to the 3′ end of the oligonucleotide described in Example 13. Preferably, the primer has a length of 20-25 bases. Clones including a sequence complementary to the biotinylated oligonucleotide were captured by incubation with streptavidin coated magnetic beads followed by magnetic selection. After capture of the positive clones, the plasmid DNA was released from the magnetic beads and converted

into double stranded DNA using a DNA polymerase such as the ThermoSequenase obtained from Amersham Pharmacia Biotech. Alternatively, protocols such as the Gene Trapper kit (Gibco BRL) may be used. The double stranded DNA was then electroporated into bacteria. The percentage of positive clones having the 5' tag oligonucleotide was estimated to typically rank between 90 and 98% using dot blot analysis.

**[0149]** Following electroporation, the libraries were ordered in 384-microtiter plates (MTP). A copy of the MTP was stored for future needs. Then the libraries were transferred into 96 MTP and sequenced as described below.

## EXAMPLE 17

Sequencing of Inserts in Selected Clones

**[0150]** Plasmid inserts were first amplified by PCR on PE 9600 thermocyclers (Perkin-Elmer), using standard SETA-A and SETA-B primers (Genset SA), AmpliTaqGold (Perkin-Elmer), dNTPs (Boehringer), buffer and cycling conditions as recommended by the Perkin-Elmer Corporation.

**[0151]** PCR products were then sequenced using automatic ABI Prism 377 sequencers (Perkin Elmer, Applied Biosystems Division, Foster City, CA). Sequencing reactions were performed using PE 9600 thermocyclers (Perkin Elmer) with standard dye-primer chemistry and ThermoSequenase (Amersham Life Science). The primers used were either T7 or 21M13 (available from Genset SA) as appropriate. The primers were labeled with the JOE, FAM, ROX and TAMRA dyes. The dNTPs and ddNTPs used in the sequencing reactions were purchased from Boehringer. Sequencing buffer, reagent concentrations and cycling conditions were as recommended by Amersham.

**[0152]** Following the sequencing reaction, the samples were precipitated with EtOH, resuspended in formamide loading buffer, and loaded on a standard 4% acrylamide gel. Electrophoresis was performed for 2.5 hours at 3000V on an ABI 377 sequencer, and the sequence data were collected and analyzed using the ABI Prism DNA Sequencing Analysis Software, version 2.1.2.

**[0153]** The sequence data from the 44 cDNA libraries made as described above were transferred to a proprietary database, where quality control and validation steps were performed. A proprietary base-caller ("Trace"), working using a Unix system automatically flagged suspect peaks, taking into account the shape of the peaks, the inter-peak resolution, and the noise level. The proprietary base-caller also performed an automatic trimming. Any stretch of 25 or fewer bases having more than 4 suspect peaks was considered unreliable and was discarded. Sequences corresponding to cloning vector or ligation oligonucleotides were automatically removed from the EST sequences. However, the resulting EST sequences may contain 1 to 5 bases belonging to the above mentioned sequences at their 5′ end. If needed, these can easily be removed on a case by case basis.

**[0154]** Thereafter, the sequences were transferred to the proprietary NETGENE™ Database for further analysis as described below.

**[0155]** Following sequencing as described above, the sequences of the 5′ ESTs were entered in a proprietary database called NETGENE™ for storage and manipulation. It will be appreciated by those skilled in the art that the data could be stored and manipulated on any medium which can be read and accessed by a computer. Computer readable media include magnetically readable media, optically readable media, or electronically readable media. For example, the computer readable media may be a hard disc, a floppy disc, a magnetic tape, CD-ROM, RAM, or ROM as well as other types of other media known to those skilled in the art.

**[0156]** In addition, the sequence data may be stored and manipulated in a variety of data processor programs in a variety of formats. For example, the sequence data may be stored as text in a word processing file, such as MicrosoftWORD or WORDPERFECT or as an ASCII file in a variety of database programs familiar to those of skill in the art, such as DB2, SYBASE, or ORACLE.

**[0157]** The computer readable media on which the sequence information is stored may be in a personal computer, a network, a server or other computer systems known to those skilled in the art. The computer or other system preferably includes the storage media described above, and a processor for accessing and manipulating the

sequence data. Once the sequence data has been stored it may be manipulated and searched to locate those stored sequences which contain a desired nucleic acid sequence or which encode a protein having a particular functional domain. For example, the stored sequence information may be compared to other known sequences to identify homologies, motifs implicated in biological function, or structural motifs.

**[0158]** Programs which may be used to search or compare the stored sequences include the MacPattern (EMBL), BLAST, and BLAST2 program series (NCBI), basic local alignment search tool programs for nucleotide (BLASTN) and peptide (BLASTX) comparisons (Altschul et al, **J. Mol. Biol. 215**: 403 (1990)) and FASTA (Pearson and Lipman, **Proc. Natl. Acad. Sci. USA, 85**: 2444 (1988)). The BLAST programs then extend the alignments on the basis of defined match and mismatch criteria.

**[0159]** Motifs which may be detected using the above programs include sequences encoding leucine zippers, helix-turn-helix motifs, glycosylation sites, ubiquitination sites, alpha helices, and beta sheets, signal sequences encoding signal peptides which direct the secretion of the encoded proteins, sequences implicated in transcription regulation such as homeoboxes, acidic stretches, enzymatic active sites, substrate binding sites, and enzymatic cleavage sites.

**[0160]** Before searching the cDNAs in the NETGENE™ database for sequence motifs of interest, cDNAs derived from mRNAs which were not of interest were identified and eliminated from further consideration as described in Example 18 below.

## EXAMPLE 18

Elimination of Undesired Sequences from Further Consideration

**[0161]** 5′ ESTs in the NETGENE™ database which were derived from undesired sequences such as transfer RNAs, ribosomal RNAs, mitochondrial RNAs, procaryotic RNAs, fungal RNAs, Alu sequences, L1 sequences, or repeat sequences were identified using the FASTA and BLASTN programs with the parameters listed in Table II.

**[0162]** To eliminate 5′ ESTs encoding tRNAs from further consideration, the 5′ EST sequences were compared to the sequences of 1190 known tRNAs obtained

from EMBL release 38, of which 100 were human. The comparison was performed using FASTA on both strands of the 5′ ESTs. Sequences having more than 80% homology over more than 60 nucleotides were identified as tRNA. Of the 144,341 sequences screened, 26 were identified as tRNAs and eliminated from further consideration.

**[0163]** To eliminate 5′ ESTs encoding rRNAs from further consideration, the 5′ EST sequences were compared to the sequences of 2497 known rRNAs obtained from EMBL release 38, of which 73 were human. The comparison was performed using BLASTN on both strands of the 5′ ESTs with the parameter S=108. Sequences having more than 80% homology over stretches longer than 40 nucleotides were identified as rRNAs. Of the 144,341 sequences screened, 3,312 were identified as rRNAs and eliminated from further consideration.

**[0164]** To eliminate 5′ ESTs encoding mtRNAs from further consideration, the 5′ EST sequences were compared to the sequences of the two known mitochondrial genomes for which the entire genomic sequences are available and all sequences transcribed from these mitochondrial genomes including tRNAs, rRNAs, and mRNAs for a total of 38 sequences. The comparison was performed using BLASTN on both strands of the 5′ ESTs with the parameter S=108. Sequences having more than 80% homology over stretches longer than 40 nucleotides were identified as mtRNAs. Of the 144,341 sequences screened, 6,110 were identified as mtRNAs and eliminated from further consideration.

**[0165]** Sequences which might have resulted from exogenous contaminants were eliminated from further consideration by comparing the 5′ EST sequences to release 46 of the EMBL bacterial and fungal divisions using BLASTN with the parameter S=144. All sequences having more than 90% homology over at least 40 nucleotides were identified as exogenous contaminants. Of the 42 cDNA libraries examined, the average percentages of procaryotic and fungal sequences contained therein were 0.2% and 0.5% respectively. Among these sequences, only one could be identified as a sequence specific to fungi. The others were either fungal or procaryotic sequences having homologies with vertebrate sequences or including repeat sequences which had not been masked during the electronic comparison.

**[0166]** In addition, the 5′ ESTs were compared to 6093 Alu sequences and 1115 L1 sequences to mask 5′ ESTs containing such repeat sequences from further consideration. 5′ ESTs including THE and MER repeats, SSTR sequences or satellite, micro-satellite, or telomeric repeats were also eliminated from further consideration. On average, 11.5% of the sequences in the libraries contained repeat sequences. Of this 11.5%, 7% contained Alu repeats, 3.3% contained L1 repeats and the remaining 1.2% were derived from the other types of repetitive sequences which were screened. These percentages are consistent with those found in cDNA libraries prepared by other groups. For example, the cDNA libraries of Adams et al. contained between 0% and 7.4% Alu repeats depending on the source of the RNA which was used to prepare the cDNA library (Adams et al., *Nature* **377**:174, 1996).

**[0167]** The sequences of those 5′ ESTs remaining after the elimination of undesirable sequences were compared with the sequences of known human mRNAs to determine the accuracy of the sequencing procedures described above.



## EXAMPLE 19

Measurement of Sequencing Accuracy by Comparison to Known Sequences

**[0168]** To further determine the accuracy of the sequencing procedure described above, the sequences of 5′ ESTs derived from known sequences were identified and compared to the known sequences. First, a FASTA analysis with overhangs shorter than 5 bp on both ends was conducted on the 5′ ESTs to identify those matching an entry in the public human mRNA database. The 6655 5′ ESTs which matched a known human mRNA were then realigned with their cognate mRNA and dynamic programming was used to include substitutions, insertions, and deletions in the list of "errors" which would be recognized. Errors occurring in the last 10 bases of the 5′ EST sequences were ignored to avoid the inclusion of spurious cloning sites in the analysis of sequencing accuracy.

**[0169]** This analysis revealed that the sequences incorporated in the NETGENE™ database had an accuracy of more than 99.5%.

**[0170]** To determine the efficiency with which the above selection procedures select cDNAs which include the 5′ ends of their corresponding mRNAs, the following analysis was performed.

## EXAMPLE 20

Determination of Efficiency of 5′ EST Selection

**[0171]** To determine the efficiency at which the above selection procedures isolated 5′ ESTs which included sequences close to the 5′ end of the mRNAs from which they were derived, the sequences of the ends of the 5′ ESTs which were derived from the elongation factor 1 subunit α and ferritin heavy chain genes were compared to the known cDNA sequences for these genes. Since the transcription start sites for the elongation factor 1 subunit α and ferritin heavy chain are well characterized, they may be used to determine the percentage of 5′ ESTs derived from these genes which included the authentic transcription start sites.

**[0172]** For both genes, more than 95% of the cDNAs included sequences close to or upstream of the 5′ end of the corresponding mRNAs.

**[0173]** To extend the analysis of the reliability of the procedures for isolating 5′ ESTs from ESTs in the NETGENE™ database, a similar analysis was conducted using a database composed of human mRNA sequences extracted from GenBank database release 97 for comparison. For those 5′ ESTs derived from mRNAs included in the GeneBank database, more than 85% had their 5′ ends close to the 5′ ends of the known sequence. As some of the mRNA sequences available in the GenBank database are deduced from genomic sequences, a 5′ end matching with these sequences will be counted as an internal match. Thus, the method used here underestimates the yield of ESTs including the authentic 5′ ends of their corresponding mRNAs.

**[0174]** The EST libraries made above included multiple 5′ ESTs derived from the same mRNA. The sequences of such 5′ ESTs were compared to one another and the longest 5′ ESTs for each mRNA were identified. Overlapping cDNAs were assembled into continuous sequences (contigs). The resulting continuous sequences were then

compared to public databases to gauge their similarity to known sequences, as described in Example 21 below.

## EXAMPLE 21

Clustering of the 5′ ESTs and Calculation of Novelty Indices for cDNA Libraries

**[0175]** For each sequenced EST library, the sequences were clustered by the 5′ end. Each sequence in the library was compared to the others with BLASTN2 (direct strand, parameters S=107). ESTs with High Scoring Segment Pairs (HSPs) at least 25 bp long, having 95% identical bases and beginning closer than 10 bp from each EST 5′ end were grouped. The longest sequence found in the cluster was used as representative of the cluster. A global clustering between libraries was then performed leading to the definition of super-contigs.

**[0176]** To assess the yield of new sequences within the EST libraries, a novelty rate (NR) was defined as: NR= 100 X (Number of new unique sequences found in the library/Total number of sequences from the library). Typically, novelty rating range between 10% and 41% depending on the tissue from which the EST library was obtained. For most of the libraries, the random sequencing of 5′ EST libraries was pursued until the novelty rate reached 20%.

**[0177]** Following characterization as described above, the collection of 5′ ESTs in NETGENE™ was screened to identify those 5′ ESTs bearing potential signal sequences as described in Example 22 below.

## EXAMPLE 22

Identification of Potential Signal Sequences in 5′ ESTs

**[0178]** The 5′ ESTs in the NETGENE™ database were screened to identify those having an uninterrupted open reading frame (ORF) longer than 45 nucleotides beginning with an ATG codon and extending to the end of the EST. Approximately half of the cDNA sequences in NETGENE™ contained such an ORF. The

ORFs of these 5′ ESTs were searched to identify potential signal motifs using slight modifications of the procedures disclosed in Von Heijne, G. A New Method for Predicting Signal Sequence Cleavage Sites. **Nucleic Acids Res**. 14:4683-4690 (1986), the disclosure of which is incorporated herein by reference. Those 5′ EST sequences encoding a 15 amino acid long stretch with a score of at least 3.5 in the Von Heijne signal peptide identification matrix were considered to possess a signal sequence. Those 5′ ESTs which matched a known human mRNA or EST sequence and had a 5′ end more than 20 nucleotides downstream of the known 5′ end were excluded from further analysis. The remaining cDNAs having signal sequences therein were included in a database called SIGNALTAG™.

**[0179]** To confirm the accuracy of the above method for identifying signal sequences, the analysis of Example 23 was performed.



## EXAMPLE 23

Confirmation of Accuracy of Identification of Potential Signal Sequences in 5′ ESTs

**[0180]** The accuracy of the above procedure for identifying signal sequences encoding signal peptides was evaluated by applying the method to the 43 amino terminal amino acids of all human SwissProt proteins. The computed Von Heijne score for each protein was compared with the known characterization of the protein as being a secreted protein or a non-secreted protein. In this manner, the number of non-secreted proteins having a score higher than 3.5 (false positives) and the number of secreted proteins having a score lower than 3.5 (false negatives) could be calculated.

**[0181]** Using the results of the above analysis, the probability that a peptide encoded by the 5′ region of the mRNA is in fact a genuine signal peptide based on its Von Heijne's score was calculated based on either the assumption that 10% of human proteins are secreted or the assumption that 20% of human proteins are secreted. The results of this analysis are shown in Figures 2 and 3.

**[0182]** Using the above method of identifying secretory proteins, 5′ ESTs for human glucagon, gamma interferon induced monokine precursor, secreted cyclophilin-

like protein, human pleiotropin, and human biotinidase precursor all of which are polypeptides which are known to be secreted, were obtained. Thus, the above method successfully identified those 5′ ESTs which encode a signal peptide.

**[0183]** To confirm that the signal peptide encoded by the 5′ ESTs actually functions as a signal peptide, the signal sequences from the 5′ ESTs may be cloned into a vector designed for the identification of signal peptides. Some signal peptide identification vectors are designed to confer the ability to grow in selective medium on host cells which have a signal sequence operably inserted into the vector. For example, to confirm that a 5′ EST encodes a genuine signal peptide, the signal sequence of the 5′ EST may be inserted upstream and in frame with a non-secreted form of the yeast invertase gene in signal peptide selection vectors such as those described in U.S. Patent No. 5,536,637, the disclosure of which is incorporated herein by reference. Growth of host cells containing signal sequence selection vectors having the signal sequence from the 5′ EST inserted therein confirms that the 5′ EST encodes a genuine signal peptide.

**[0184]** Alternatively, the presence of a signal peptide may be confirmed by cloning the extended cDNAs obtained using the ESTs into expression vectors such as pXT1 (as described below), or by constructing promoter-signal sequence-reporter gene vectors which encode fusion proteins between the signal peptide and an assayable reporter protein. After introduction of these vectors into a suitable host cell, such as COS cells or NIH 3T3 cells, the growth medium may be harvested and analyzed for the presence of the secreted protein. The medium from these cells is compared to the medium from cells containing vectors lacking the signal sequence or extended cDNA insert to identify vectors which encode a functional signal peptide or an authentic secreted protein.

**[0185]** Those 5′ ESTs which encoded a signal peptide, as determined by the method of Example 22 above, were further grouped into four categories based on their homology to known sequences. The categorization of the 5′ ESTs is described in Example 24 below.

## EXAMPLE 24

Categorization of 5′ ESTs Encoding a Signal Peptide

**[0186]** Those 5′ ESTs having a sequence not matching any known vertebrate sequence nor any publicly available EST sequence were designated "new." Of the sequences in the SIGNALTAG™ database, 947 of the 5′ ESTs having a Von Heijne's score of at least 3.5 fell into this category.

**[0187]** Those 5′ ESTs having a sequence not matching any vertebrate sequence but matching a publicly known EST were designated "EST-ext", provided that the known EST sequence was extended by at least 40 nucleotides in the 5′ direction. Of the sequences in the SIGNALTAG™ database, 150 of the 5′ ESTs having a Von Heijne's score of at least 3.5 fell into this category.

**[0188]** Those ESTs not matching any vertebrate sequence but matching a publicly known EST without extending the known EST by at least 40 nucleotides in the 5′ direction were designated "EST." Of the sequences in the SIGNALTAG™ database, 599 of the 5′ ESTs having a Von Heijne's score of at least 3.5 fell into this category.

**[0189]** Those 5′ ESTs matching a human mRNA sequence but extending the known sequence by at least 40 nucleotides in the 5′ direction were designated "VERT-ext." Of the sequences in the SIGNALTAG™ database, 23 of the 5′ ESTs having a Von Heijne's score of at least 3.5 fell into this category. Included in this category was a 5′ EST which extended the known sequence of the human translocase mRNA by more than 200 bases in the 5′ direction. A 5′ EST which extended the sequence of a human tumor suppressor gene in the 5′ direction was also identified.

**[0190]** Figure 4 shows the distribution of 5′ ESTs in each category and the number of 5′ ESTs in each category having a given minimum von Heijne's score.

**[0191]** Each of the 5′ ESTs was categorized based on the tissue from which its corresponding mRNA was obtained, as described below in Example 25.

## EXAMPLE 25

Categorization of Expression Patterns

**[0192]** Figure 5 shows the tissues from which the mRNAs corresponding to the 5′ ESTs in each of the above described categories were obtained.

**[0193]** In addition to categorizing the 5′ ESTs by the tissue from which the cDNA library in which they were first identified was obtained, the spatial and temporal expression patterns of the mRNAs corresponding to the 5′ ESTs, as well as their expression levels, may be determined as described in Example 26 below. Characterization of the spatial and temporal expression patterns and expression levels of these mRNAs is useful for constructing expression vectors capable of producing a desired level of gene product in a desired spatial or temporal manner, as will be discussed in more detail below.

**[0194]** In addition, 5′ ESTs whose corresponding mRNAs are associated with disease states may also be identified. For example, a particular disease may result from lack of expression, over expression, or under expression of an mRNA corresponding to a 5′ EST. By comparing mRNA expression patterns and quantities in samples taken from healthy individuals with those from individuals suffering from a particular disease, 5′ ESTs responsible for the disease may be identified.

**[0195]** It will be appreciated that the results of the above characterization procedures for 5′ ESTs also apply to extended cDNAs (obtainable as described below) which contain sequences adjacent to the 5′ ESTs. It will also be appreciated that if it is desired to defer characterization until extended cDNAs have been obtained rather than characterizing the ESTs themselves, the above characterization procedures can be applied to characterize the extended cDNAs after their isolation.

## EXAMPLE 26

Evaluation of Expression Levels and Patterns of mRNAs

Corresponding to 5′ ESTs or Extended cDNAs

**[0196]** Expression levels and patterns of mRNAs corresponding to 5′ ESTs or extended cDNAs (obtainable as described below) may be analyzed by solution hybridization with long probes as described in International Patent Application No. WO 97/05277, the entire contents of which are hereby incorporated by reference. Briefly, a 5′ EST, extended cDNA, or fragment thereof corresponding to the gene encoding the mRNA to be characterized is inserted at a cloning site immediately downstream of a bacteriophage (T3, T7 or SP6) RNA polymerase promoter to produce antisense RNA. Preferably, the 5′ EST or extended cDNA has 100 or more nucleotides. The plasmid is linearized and transcribed in the presence of ribonucleotides comprising modified ribonucleotides (i.e. biotin-UTP and DIG-UTP). An excess of this doubly labeled RNA is hybridized in solution with mRNA isolated from cells or tissues of interest. The hybridizations are performed under standard stringent conditions (40-50°C for 16 hours in an 80% formamide, 0.4 M NaCl buffer, pH 7-8). The unhybridized probe is removed by digestion with ribonucleases specific for single-stranded RNA (i.e. RNases CL3, T1, Phy M, U2 or A). The presence of the biotin-UTP modification enables capture of the hybrid on a microtitration plate coated with streptavidin. The presence of the DIG modification enables the hybrid to be detected and quantified by ELISA using an anti-DIG antibody coupled to alkaline phosphatase.

**[0197]** The 5′ ESTs, extended cDNAs, or fragments thereof may also be tagged with nucleotide sequences for the serial analysis of gene expression (SAGE) as disclosed in UK Patent Application No. 2 305 241 A, the entire contents of which are incorporated by reference. In this method, cDNAs are prepared from a cell, tissue, organism or other source of nucleic acid for which it is desired to determine gene expression patterns. The resulting cDNAs are separated into two pools. The cDNAs in each pool are cleaved with a first restriction endonuclease, called an "anchoring enzyme," having a recognition site which is likely to be present at least once in most cDNAs. The fragments which contain the 5′ or 3′ most region of the cleaved cDNA are isolated by

binding to a capture medium such as streptavidin coated beads. A first oligonucleotide linker having a first sequence for hybridization of an amplification primer and an internal restriction site for a "tagging endonuclease" is ligated to the digested cDNAs in the first pool. Digestion with the second endonuclease produces short "tag" fragments from the cDNAs.

**[0198]** A second oligonucleotide having a second sequence for hybridization of an amplification primer and an internal restriction site is ligated to the digested cDNAs in the second pool. The cDNA fragments in the second pool are also digested with the "tagging endonuclease" to generate short "tag" fragments derived from the cDNAs in the second pool. The "tags" resulting from digestion of the first and second pools with the anchoring enzyme and the tagging endonuclease are ligated to one another to produce "ditags." In some embodiments, the ditags are concatamerized to produce ligation products containing from 2 to 200 ditags. The tag sequences are then determined and compared to the sequences of the 5′ ESTs or extended cDNAs to determine which 5′ ESTs or extended cDNAs are expressed in the cell, tissue, organism, or other source of nucleic acids from which the tags were derived. In this way, the expression pattern of the 5′ ESTs or extended cDNAs in the cell, tissue, organism, or other source of nucleic acids is obtained.

**[0199]** Quantitative analysis of gene expression may also be performed using arrays. As used herein, the term array means a one dimensional, two dimensional, or multidimensional arrangement of full length cDNAs (i.e. extended cDNAs which include the coding sequence for the signal peptide, the coding sequence for the mature protein, and a stop codon), extended cDNAs, 5′ ESTs or fragments of the full length cDNAs, extended cDNAs, or 5′ ESTs of sufficient length to permit specific detection of gene expression. Preferably, the fragments are at least 15 nucleotides in length. More preferably, the fragments are at least 100 nucleotides in length. More preferably, the fragments are more than 100 nucleotides in length. In some embodiments the fragments may be more than 500 nucleotides in length.

**[0200]** For example, quantitative analysis of gene expression may be performed with full length cDNAs, extended cDNAs, 5′ ESTs, or fragments thereof in a complementary DNA microarray as described by Schena et al. (*Science* **270**:467-470,

1995; *Proc. Natl. Acad. Sci. U.S.A.* **93**:10614-10619, 1996). Full length cDNAs, extended cDNAs, 5′ ESTs or fragments thereof are amplified by PCR and arrayed from 96-well microtiter plates onto silylated microscope slides using high-speed robotics. Printed arrays are incubated in a humid chamber to allow rehydration of the array elements and rinsed, once in 0.2% SDS for 1 min, twice in water for 1 min and once for 5 min in sodium borohydride solution. The arrays are submerged in water for 2 min at 95°C, transferred into 0.2% SDS for 1 min, rinsed twice with water, air dried and stored in the dark at 25°C.

**[0201]** Cell or tissue mRNA is isolated or commercially obtained and probes are prepared by a single round of reverse transcription. Probes are hybridized to 1 $cm^2$ microarrays under a 14 x 14 mm glass coverslip for 6-12 hours at 60°C. Arrays are washed for 5 min at 25°C in low stringency wash buffer (1 x SSC/0.2% SDS), then for 10 min at room temperature in high stringency wash buffer (0.1 x SSC/0.2% SDS). Arrays are scanned in 0.1 x SSC using a fluorescence laser scanning device fitted with a custom filter set. Accurate differential expression measurements are obtained by taking the average of the ratios of two independent hybridizations.

**[0202]** Quantitative analysis of the expression of genes may also be performed with full length cDNAs, extended cDNAs, 5′ ESTs, or fragments thereof in complementary DNA arrays as described by Pietu et al. (Genome Research 6:492-503, 1996). The full length cDNAs, extended cDNAs, 5′ ESTs or fragments thereof are PCR amplified and spotted on membranes. Then, mRNAs originating from various tissues or cells are labeled with radioactive nucleotides. After hybridization and washing in controlled conditions, the hybridized mRNAs are detected by phospho-imaging or autoradiography. Duplicate experiments are performed and a quantitative analysis of differentially expressed mRNAs is then performed.

**[0203]** Alternatively, expression analysis of the 5′ ESTs or extended cDNAs can be done through high density nucleotide arrays as described by Lockhart et al. (Nature Biotechnology 14: 1675-1680, 1996) and Sosnowsky et al. (Proc. Natl. Acad. Sci. 94:1119-1123, 1997). Oligonucleotides of 15-50 nucleotides corresponding to sequences of the 5′ ESTs or extended cDNAs are synthesized directly on the chip (Lockhart et al.,

*supra*) or synthesized and then addressed to the chip (Sosnowski et al., *supra*). Preferably, the oligonucleotides are about 20 nucleotides in length.

**[0204]** cDNA probes labeled with an appropriate compound, such as biotin, digoxigenin or fluorescent dye, are synthesized from the appropriate mRNA population and then randomly fragmented to an average size of 50 to 100 nucleotides. The said probes are then hybridized to the chip. After washing as described in Lockhart et al., *supra* and application of different electric fields (Sosnowsky et al., Proc. Natl. Acad. Sci. 94:1119-1123)., the dyes or labeling compounds are detected and quantified. Duplicate hybridizations are performed. Comparative analysis of the intensity of the signal originating from cDNA probes on the same target oligonucleotide in different cDNA samples indicates a differential expression of the mRNA corresponding to the 5′ EST or extended cDNA from which the oligonucleotide sequence has been designed.



## III. Use of 5′ ESTs to Clone Extended cDNAs and to Clone the Corresponding Genomic DNAs

**[0205]** Once 5′ ESTs which include the 5′ end of the corresponding mRNAs have been selected using the procedures described above, they can be utilized to isolate extended cDNAs which contain sequences adjacent to the 5′ ESTs. The extended cDNAs may include the entire coding sequence of the protein encoded by the corresponding mRNA, including the authentic translation start site, the signal sequence, and the sequence encoding the mature protein remaining after cleavage of the signal peptide. Such extended cDNAs are referred to herein as "full length cDNAs." Alternatively, the extended cDNAs may include only the sequence encoding the mature protein remaining after cleavage of the signal peptide, or only the sequence encoding the signal peptide.

**[0206]** Example 27 below describes a general method for obtaining extended cDNAs. Example 28 below describes the cloning and sequencing of several extended cDNAs, including extended cDNAs which include the entire coding sequence and authentic 5′ end of the corresponding mRNA for several secreted proteins.

**[0207]** The methods of Examples 27, 28, and 29 can also be used to obtain extended cDNAs which encode less than the entire coding sequence of the secreted proteins encoded by the genes corresponding to the 5′ ESTs. In some embodiments, the extended cDNAs isolated using these methods encode at least 10 amino acids of one of the proteins encoded by the sequences of SEQ ID NOs: 40-84 and 130-154. In further embodiments, the extended cDNAs encode at least 20 amino acids of the proteins encoded by the sequences of SEQ ID NOs: 40-84 and 130-154. In further embodiments, the extended cDNAs encode at least 30 amino amino acids of the sequences of SEQ ID NOs: 40-84 and 130-154. In a preferred embodiment, the extended cDNAs encode a full length protein sequence, which includes the protein coding sequences of SEQ ID NOs: 40-84 and 130-154.



## EXAMPLE 27

General Method for Using 5′ ESTs to Clone and Sequence Extended cDNAs

**[0208]** The following general method has been used to quickly and efficiently isolate extended cDNAs including sequence adjacent to the sequences of the 5′ ESTs used to obtain them. This method may be applied to obtain extended cDNAs for any 5′ EST in the NETGENE™ database, including those 5′ ESTs encoding secreted proteins. The method is summarized in Figure 6.

1. Obtaining Extended cDNAs

a) First strand synthesis

**[0209]** The method takes advantage of the known 5′ sequence of the mRNA. A reverse transcription reaction is conducted on purified mRNA with a poly 14dT primer containing a 49 nucleotide sequence at its 5′ end allowing the addition of a known sequence at the end of the cDNA which corresponds to the 3′ end of the mRNA. For example, the primer may have the following sequence: 5′-ATC GTT GAG ACT CGT ACC AGC AGA GTC ACG AGA GAG ACT ACA CGG TAC TGG TTT TTT TTT TTT TTVN -3′ (SEQ ID NO:14). Those skilled in the art will appreciate that other sequences may also be added to the poly dT sequence and used to prime the first strand synthesis.

Using this primer and a reverse transcriptase such as the Superscript II (Gibco BRL) or Rnase H Minus M-MLV (Promega) enzyme, a reverse transcript anchored at the 3' polyA site of the RNAs is generated.

**[0210]** After removal of the mRNA hybridized to the first cDNA strand by alkaline hydrolysis, the products of the alkaline hydrolysis and the residual poly dT primer are eliminated with an exclusion column such as an AcA34 (Biosepra) matrix as explained in Example 11.

b) Second strand synthesis

**[0211]** A pair of nested primers on each end is designed based on the known 5' sequence from the 5' EST and the known 3' end added by the poly dT primer used in the first strand synthesis. Software used to design primers are either based on GC content and melting temperatures of oligonucleotides, such as OSP (Illier and Green, *PCR Meth. Appl.* **1**:124-128, 1991), or based on the octamer frequency disparity method (Griffais et al., *Nucleic Acids Res.* **19**: 3887-3891, 1991 such as PC-Rare (http://bioinformatics.weizmann.ac.il/software/PC-Rare/doc/manuel.html).

**[0212]** Preferably, the nested primers at the 5' end are separated from one another by four to nine bases. The 5' primer sequences may be selected to have melting temperatures and specificities suitable for use in PCR.

**[0213]** Preferably, the nested primers at the 3' end are separated from one another by four to nine bases. For example, the nested 3' primers may have the following sequences: (5'- CCA GCA GAG TCA CGA GAG AGA CTA CAC GG -3'(SEQ ID NO:15), and 5'- CAC GAG AGA GAC TAC ACG GTA CTG G -3' (SEQ ID NO:16). These primers were selected because they have melting temperatures and specificities compatible with their use in PCR. However, those skilled in the art will appreciate that other sequences may also be used as primers.

**[0214]** The first PCR run of 25 cycles is performed using the Advantage Tth Polymerase Mix (Clontech) and the outer primer from each of the nested pairs. A second 20 cycle PCR using the same enzyme and the inner primer from each of the nested pairs is then performed on 1/2500 of the first PCR product. Thereafter, the primers and nucleotides are removed.

2. Sequencing of Full Length Extended cDNAs or Fragments Thereof

**[0215]** Due to the lack of position constraints on the design of 5' nested primers compatible for PCR use using the OSP software, amplicons of two types are obtained. Preferably, the second 5' primer is located upstream of the translation initiation codon thus yielding a nested PCR product containing the whole coding sequence. Such a full length extended cDNA undergoes a direct cloning procedure as described in section a below. However, in some cases, the second 5' primer is located downstream of the translation initiation codon, thereby yielding a PCR product containing only part of the ORF. Such incomplete PCR products are submitted to a modified procedure described in section b below.

a) Nested PCR products containing complete ORFs

**[0216]** When the resulting nested PCR product contains the complete coding sequence, as predicted from the 5'EST sequence, it is cloned in an appropriate vector such as pED6dpc2, as described in section 3.

b) Nested PCR products containing incomplete ORFs

**[0217]** When the amplicon does not contain the complete coding sequence, intermediate steps are necessary to obtain both the complete coding sequence and a PCR product containing the full coding sequence. The complete coding sequence can be assembled from several partial sequences determined directly from different PCR products as described in the following section.

**[0218]** Once the full coding sequence has been completely determined, new primers compatible for PCR use are designed to obtain amplicons containing the whole coding region. However, in such cases, 3' primers compatible for PCR use are located inside the 3' UTR of the corresponding mRNA, thus yielding amplicons which lack part of this region, i.e. the polyA tract and sometimes the polyadenylation signal, as illustrated in figure 6. Such full length extended cDNAs are then cloned into an appropriate vector as described in section 3.

c) Sequencing extended cDNAs

**[0219]** Sequencing of extended cDNAs can be performed using a Die Terminator approach with the AmpliTaq DNA polymerase FS kit available from Perkin Elmer.

**[0220]** In order to sequence PCR fragments, primer walking is performed using software such as OSP to choose primers and automated computer software such as ASMG (Sutton et al., *Genome Science Technol.* **1**: 9-19, 1995) to construct contigs of walking sequences including the initial 5' tag using minimum overlaps of 32 nucleotides. Preferably, primer walking is performed until the sequences of full length cDNAs are obtained.

**[0221]** Completion of the sequencing of a given extended cDNA fragment is assessed as follows. Since sequences located after a polyA tract are difficult to determine precisely in the case of uncloned products, sequencing and primer walking processes for PCR products are interrupted when a polyA tract is identified in extended cDNAs obtained as described in case b. The sequence length is compared to the size of the nested PCR product obtained as described above. Due to the limited accuracy of the determination of the PCR product size by gel electrophoresis, a sequence is considered complete if the size of the obtained sequence is at least 70 % the size of the first nested PCR product. If the length of the sequence determined from the computer analysis is not at least 70% of the length of the nested PCR product, these PCR products are cloned and the sequence of the insertion is determined. When Northern blot data are available, the size of the mRNA detected for a given PCR product is used to finally assess that the sequence is complete. Sequences which do not fulfill the above criteria are discarded and will undergo a new isolation procedure.

**[0222]** Sequence data of all extended cDNAs are then transferred to a proprietary database, where quality controls and validation steps are carried out as described in example 15.

3. Cloning of Full Length Extended cDNAs

**[0223]** The PCR product containing the full coding sequence is then cloned in an appropriate vector. For example, the extended cDNAs can be cloned into the

expression vector pED6dpc2 (DiscoverEase, Genetics Institute, Cambridge, MA) as follows. The structure of pED6dpc2 is shown in Figure 7. pED6dpc2 vector DNA is prepared with blunt ends by performing an EcoRI digestion followed by a fill in reaction. The blunt ended vector is dephosphorylated. After removal of PCR primers and ethanol precipitation, the PCR product containing the full coding sequence or the extended cDNA obtained as described above is phosphorylated with a kinase subsequently removed by phenol-Sevag extraction and precipitation. The double stranded extended cDNA is then ligated to the vector and the resulting expression plasmid introduced into appropriate host cells.

**[0224]** Since the PCR products obtained as described above are blunt ended molecules that can be cloned in either direction, the orientation of several clones for each PCR product is determined. Then, 4 to 10 clones are ordered in microtiter plates and subjected to a PCR reaction using a first primer located in the vector close to the cloning site and a second primer located in the portion of the extended cDNA corresponding to the 3' end of the mRNA. This second primer may be the antisense primer used in anchored PCR in the case of direct cloning (case a) or the antisense primer located inside the 3'UTR in the case of indirect cloning (case b). Clones in which the start codon of the extended cDNA is operably linked to the promoter in the vector so as to permit expression of the protein encoded by the extended cDNA are conserved and sequenced. In addition to the ends of cDNA inserts, approximately 50 bp of vector DNA on each side of the cDNA insert are also sequenced.

**[0225]** The cloned PCR products are then entirely sequenced according to the aforementioned procedure. In this case, contig assembly of long fragments is then performed on walking sequences that have already contigated for uncloned PCR products during primer walking. Sequencing of cloned amplicons is complete when the resulting contigs include the whole coding region as well as overlapping sequences with vector DNA on both ends.

4. Computer Analysis of Full Length Extended cDNA

**[0226]** Sequences of all full length extended cDNAs may then be subjected to further analysis as described below and using the parameters found in Table II with the following modifications. For screening of miscellaneous subdivisions

of Genbank, FASTA was used instead of BLASTN and 15 nucleotide of homology was the limit instead of 17. For Alu detection, BLASTN was used with the following parameters: S=72; identity=70%; and length = 40 nucleotides. Polyadenylation signal and polyA tail which were not search for the 5' ESTs were searched. For polyadenylation signal detection the signal (AATAAA) was searched with one permissible mismatch in the last fifty nucleotides preceding the 5' end of the polyA. For the polyA, a stretch of 8 amino acids in the last 20 nucleotides of the sequence was searched with BLAST2N in the sense strand with the following parameters (W=6, S=10, E=1000, and identity=90%). Finally, patented sequences and ORF homologies were searched using, respectively, BLASTN and BLASTP on GenSEQ (Derwent's database of patented nucleotide sequences) and SWISSPROT for ORFs with the following parameters (W=8 and B=10). Before examining the extended full length cDNAs for sequences of interest, extended cDNAs which are not of interest are searched as follows.

a) Elimination of undesired sequences

**[0227]** Although 5'ESTs were checked to remove contaminants sequences as described in Example 18, a last verification was carried out to identify extended cDNAs sequences derived from undesired sequences such as vector RNAs, transfer RNAs, ribosomal rRNAs, mitochondrial RNAs, prokaryotic RNAs and fungal RNAs using the FASTA and BLASTN programs on both strands of extended cDNAs as described below.

**[0228]** To identify the extended cDNAs encoding vector RNAs, extended cDNAs are compared to the known sequences of vector RNA using the FASTA program. Sequences of extended cDNAs with more than 90% homology over stretches of 15 nucleotides are identified as vector RNA.

**[0229]** To identify the extended cDNAs encoding tRNAs, extended cDNA sequences were compared to the sequences of 1190 known tRNAs obtained from EMBL release 38, of which 100 were human. Sequences of extended cDNAs having more than 80% homology over 60 nucleotides using FASTA were identified as tRNA.

**[0230]** To identify the extended cDNAs encoding rRNAs, extended cDNA sequences were compared to the sequences of 2497 known rRNAs obtained from EMBL release 38, of which 73 were human. Sequences of extended cDNAs having more than 80% homology over stretches longer than 40 nucleotides using BLASTN were identified as rRNAs.

**[0231]** To identify the extended cDNAs encoding mtRNAs, extended cDNA sequences were compared to the sequences of the two known mitochondrial genomes for which the entire genomic sequences are available and all sequences transcribed from these mitochondrial genomes including tRNAs, rRNAs, and mRNAs for a total of 38 sequences. Sequences of extended cDNAs having more than 80% homology over stretches longer than 40 nucleotides using BLASTN were identified as mtRNAs.

**[0232]** Sequences which might have resulted from other exogenous contaminants were identified by comparing extended cDNA sequences to release 105 of Genbank bacterial and fungal divisions. Sequences of extended cDNAs having more than 90% homology over 40 nucleotides using BLASTN were identified as exogenous prokaryotic or fungal contaminants.

**[0233]** In addition, extended cDNAs were searched for different repeat sequences, including Alu sequences, L1 sequences, THE and MER repeats, SSTR sequences or satellite, micro-satellite, or telomeric repeats. Sequences of extended cDNAs with more than 70% homology over 40 nucleotide stretches using BLASTN were identified as repeat sequences and masked in further identification procedures. In addition, clones showing extensive homology to repeats , i.e., matches of either more than 50 nucleotides if the homology was at least 75% or more than 40 nucleotides if the homology was at least 85% or more than 30 nucleotides if the homology was at least 90%, were flagged.

b) Identification of structural features

**[0234]** Structural features, e.g. polyA tail and polyadenylation signal, of the sequences of full length extended cDNAs are subsequently determined as follows.

**[0235]** A polyA tail is defined as a homopolymeric stretch of at least 11 A with at most one alternative base within it. The polyA tail search is restricted to the

last 20 nt of the sequence and limited to stretches of 11 consecutive A's because sequencing reactions are often not readable after such a polyA stretch. Stretches with 100% homology over 6 nucleotides are identified as polyA tails.

**[0236]** To search for a polyadenylation signal, the polyA tail is clipped from the full-length sequence. The 50 bp preceding the polyA tail are first searched for the canonic polyadenylation AAUAAA signal and, if the canonic signal is not detected, for the alternative AUUAAA signal (Sheets et al., Nuc. Acids Res. 18: 5799-5805, 1990). If neither of these consensus polyadenylation signals is found, the canonic motif is searched again allowing one mismatch to account for possible sequencing errors. More than 85 % of identified polyadenylation signals of either type actually ends 10 to 30 bp from the polyA tail. Alternative AUUAAA signals represents approximately 15 % of the total number of identified polyadenylation signals.

**[0237]** To search for a polyadenylation signal, the polyA tail is clipped from the full-length sequence. The 50 bp preceding the polyA tail are searched for the canonic polyadenylation AAUAAA signal allowing one mismatch to account for possible sequencing errors and known variation in the canonical sequence of the polyadenylation signal.

c) Identification of functional features

**[0238]** Functional features, e.g. ORFs and signal sequences, of the sequences of full length extended cDNAs were subsequently determined as follows.

**[0239]** The 3 upper strand frames of extended cDNAs are searched for ORFs defined as the maximum length fragments beginning with a translation initiation codon and ending with a stop codon. ORFs encoding at least 20 amino acids are preferred.

**[0240]** Each found ORF is then scanned for the presence of a signal peptide in the first 50 amino-acids or, where appropriate, within shorter regions down to 20 amino acids or less in the ORF, using the matrix method of von Heijne (Nuc. Acids Res. **14**: 4683-4690 (1986)), the disclosure of which is incorporated herein by reference and the modification described in Example 22.

d) Homology to either nucleotidic or proteic sequences

**[0241]** Sequences of full length extended cDNAs are then compared to known sequences on a nucleotidic or proteic basis.

**[0242]** Sequences of full length extended cDNAs are compared to the following known nucleic acid sequences: vertebrate sequences, EST sequences , patented sequences and recently identified sequences available at the time of filing the priority documents. Full length cDNA sequences are also compared to the sequences of a private database (Genset internal sequences) in order to find sequences that have already been identified by applicants. Sequences of full length extended cDNAs with more than 90% homology over 30 nucleotides using either BLASTN or BLAST2N as indicated in Table III are identified as sequences that have already been described. Matching vertebrate sequences are subsequently examined using FASTA; full length extended cDNAs with more than 70% homology over 30 nucleotides are identified as sequences that have already been described.

**[0243]** ORFs encoded by full length extended cDNAs as defined in section c) are subsequently compared to known amino acid sequences found in public databases using Swissprot, PIR and Genptept releases available at the time of filing the priority documents for the present application. These analyses were performed using BLASTP with the parameter W=8 and allowing a maximum of 10 matches. Sequences of full length extended cDNAs showing extensive homology to known protein sequences are recognized as already identified proteins.

**[0244]** In addition, the three-frame conceptual translation products of the top strand of full length extended cDNAs are compared to publicly known amino acid sequences of Swissprot using BLASTX with the parameter E=0.001. Sequences of full length extended cDNAs with more than 70% homology over 30 amino acid stretches are detected as already identified proteins.

**[0245]** As used herein the term "cDNA codes of SEQ ID NOs. 40-84 and 130-154" encompasses the nucleotide sequences of SEQ ID NOs. 40-84 and 130-154, fragments of SEQ ID NOs. 40-84 and 130-154, nucleotide sequences homologous to SEQ ID NOs. 40-84 and 130-154 or homologous to fragments of SEQ ID NOs. 40-84

and 130-154, and sequences complementary to all of the preceding sequences. The fragments include portions of SEQ ID NOs. 40-84 and 130-154 comprising at least 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 300, 400, or 500 consecutive nucleotides of SEQ ID NOs. 40-84 and 130-154. Preferably, the fragments are novel fragments. Homologous sequences and fragments of SEQ ID NOs. 40-84 and 130-154 refer to a sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, or 75% homology to these sequences. Homology may be determined using any of the computer programs and parameters described herein, including BLAST2N with the default parameters or with any modified parameters. Homologous sequences also include RNA sequences in which uridines replace the thymines in the cDNA codes of SEQ ID NOs. 40-84 and 130-154. The homologous sequences may be obtained using any of the procedures described herein or may result from the correction of a sequencing error as described above. It will be appreciated that the cDNA codes of SEQ ID NOs. 40-84 and 130-154 can be represented in the traditional single character format (See the inside back cover of Starrier, Lubert. *Biochemistry*, 3rd edition. W. H Freeman & Co., New York.) or in any other format which records the identity of the nucleotides in a sequence.

**[0246]** As used herein the term "polypeptide codes of SEQ ID NOS. 85-129 and 155-179" encompasses the polypeptide sequence of SEQ ID NOs. 85-129 and 155-179 which are encoded by the extended cDNAs of SEQ ID NOs. 40-84 and 130-154, polypeptide sequences homologous to the polypeptides of SEQ ID NOS. 85-129 and 155-179, or fragments of any of the preceding sequences. Homologous polypeptide sequences refer to a polypeptide sequence having at least 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75% homology to one of the polypeptide sequences of SEQ ID NOS. 85-129 and 155-179. Homology may be determined using any of the computer programs and parameters described herein, including FASTA with the default parameters or with any modified parameters. The homologous sequences may be obtained using any of the procedures described herein or may result from the correction of a sequencing error as described above. The polypeptide fragments comprise at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, or 150 consecutive amino acids of the polypeptides of SEQ ID NOS. 85-129 and 155-179. Preferably, the fragments are novel fragments. It will be appreciated that the polypeptide codes of the SEQ ID NOS. 85-129 and 155-179 can be represented in the

traditional single character format or three letter format (See the inside back cover of Starrier, Lubert. *Biochemistry*, 3rd edition. W. H Freeman & Co., New York.) or in any other format which relates the identity of the polypeptides in a sequence.

**[0247]** It will be appreciated by those skilled in the art that the cDNA codes of SEQ ID NOs. 40-84 and 130-154 and polypeptide codes of SEQ ID NOS. 85-129 and 155-179 can be stored, recorded, and manipulated on any medium which can be read and accessed by a computer. As used herein, the words "recorded" and "stored" refer to a process for storing information on a computer medium. A skilled artisan can readily adopt any of the presently known methods for recording information on a computer readable medium to generate manufactures comprising one or more of the cDNA codes of SEQ ID NOs. 40-84 and 130-154, one or more of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179. Another aspect of the present invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, 20, 25, 30, or 50 cDNA codes of SEQ ID NOs. 40-84 and 130-154. Another aspect of the present invention is a computer readable medium having recorded thereon at least 2, 5, 10, 15, 20, 25, 30, or 50 polypeptide codes of SEQ ID NOS. 85-129 and 155-179.

**[0248]** Computer readable media include magnetically readable media, optically readable media, electronically readable media and magnetic/optical media. For example, the computer readable media may be a hard disc, a floppy disc, a magnetic tape, CD-ROM, DVD, RAM, or ROM as well as other types of other media known to those skilled in the art.

**[0249]** Embodiments of the present invention include systems, particularly computer systems which contain the sequence information described herein. As used herein, "a computer system" refers to the hardware components, software components, and data storage components used to analyze the nucleotide sequences of the cDNA codes of SEQ ID NOs. 40-84 and 130-154, or the amino acid sequences of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179. The computer system preferably includes the computer readable media described above, and a processor for accessing and manipulating the sequence data.

**[0250]** Preferably, the computer is a general purpose system that comprises a central processing unit (CPU), one or more data storage components for storing data, and

one or more data retrieving devices for retrieving the data stored on the data storage components. A skilled artisan can readily appreciate that any one of the currently available computer systems are suitable.

**[0251]** In one particular embodiment, the computer system includes a processor connected to a bus which is connected to a main memory (preferably implemented as RAM) and one or more data storage devices, such as a hard drive and/or other computer readable media having data recorded thereon. In some embodiments, the computer system further includes one or more data retrieving devices for reading the data stored on the data storage components. The data retrieving device may represent, for example, a floppy disk drive, a compact disk drive, a magnetic tape drive, etc. In some embodiments, the data storage component is a removable computer readable medium such as a floppy disk, a compact disk, a magnetic tape, etc. containing control logic and/or data recorded thereon. The computer system may advantageously include or be programmed by appropriate software for reading the control logic and/or the data from the data storage component once inserted in the data retrieving device. Software for accessing and processing the nucleotide sequences of the cDNA codes of SEQ ID NOs. 40-84 and 130-154, or the amino acid sequences of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179 (such as search tools, compare tools, and modeling tools etc.) may reside in main memory during execution.

**[0252]** In some embodiments, the computer system may further comprise a sequence comparer for comparing the above-described cDNA codes of SEQ ID NOs. 40-84 and 130-154 or polypeptide codes of SEQ ID NOS. 85-129 and 155-179 stored on a computer readable medium to reference nucleotide or polypeptide sequences stored on a computer readable medium. A "sequence comparer" refers to one or more programs which are implemented on the computer system to compare a nucleotide or polypeptide sequence with other nucleotide or polypeptide sequences and/or compounds including but not limited to peptides, peptidomimetics, and chemicals stored within the data storage means. For example, the sequence comparer may compare the nucleotide sequences of the cDNA codes of SEQ ID NOs. 40-84 and 130-154, or the amino acid sequences of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179 stored on a computer readable medium to reference sequences stored on a computer readable medium to identify

homologies, motifs implicated in biological function, or structural motifs. The various sequence comparer programs identified elsewhere in this patent specification are particularly contemplated for use in this aspect of the invention.

**[0253]** Accordingly, one aspect of the present invention is a computer system comprising a processor, a data storage device having stored thereon a cDNA code of SEQ ID NOs. 40-84 and 130-154 or a polypeptide code of SEQ ID NOS. 85-129 and 155-179, a data storage device having retrievably stored thereon reference nucleotide sequences or polypeptide sequences to be compared to the cDNA code of SEQ ID NOs. 40-84 and 130-154 or polypeptide code of SEQ ID NOS. 85-129 and 155-179 and a sequence comparer for conducting the comparison. The sequence comparer may indicate a homology level between the sequences compared or identify structural motifs in the above described cDNA code of SEQ ID NOs. 40-84 and 130-154 and polypeptide codes of SEQ ID NOS. 85-129 and 155-179 or it may identify structural motifs in sequences which are compared to these cDNA codes and polypeptide codes. In some embodiments, the data storage device may have stored thereon the sequences of at least 2, 5, 10, 15, 20, 25, 30, or 50 of the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or polypeptide codes of SEQ ID NOS. 85-129 and 155-179.

**[0254]** Another aspect of the present invention is a method for determining the level of homology between a cDNA code of SEQ ID NOs. 40-84 and 130-154 and a reference nucleotide sequence, comprising the steps of reading the cDNA code and the reference nucleotide sequence through the use of a computer program which determines homology levels and determining homology between the cDNA code and the reference nucleotide sequence with the computer program. The computer program may be any of a number of computer programs for determining homology levels, including those specifically enumerated below, including BLAST2N with the default parameters or with any modified parameters. The method may be implemented using the computer systems described above. The method may also be performed by reading 2, 5, 10, 15, 20, 25, 30, or 50 of the above described cDNA codes of SEQ ID NOs. 40-84 and 130-154 through use of the computer program and determining homology between the cDNA codes and reference nucleotide sequences.

**[0255]** Alternatively, the computer program may be a computer program which compares the nucleotide sequences of the cDNA codes of the present invention, to reference nucleotide sequences in order to determine whether the cDNA code of SEQ ID NOs. 40-84 and 130-154 differs from a reference nucleic acid sequence at one or more positions. Optionally such a program records the length and identity of inserted, deleted or substituted nucleotides with respect to the sequence of either the reference polynucleotide or the cDNA code of SEQ ID NOs. 40-84 and 130-154. In one embodiment, the computer program may be a program which determines whether the nucleotide sequences of the cDNA codes of SEQ ID NOs. 40-84 and 130-154 contain a single nucleotide polymorphism (SNP) with respect to a reference nucleotide sequence. This single nucleotide polymorphism may comprise a single base substitution, insertion, or deletion.

**[0256]** Another aspect of the present invention is a method for determining the level of homology between a polypeptide code of SEQ ID NOS. 85-129 and 155-179 and a reference polypeptide sequence, comprising the steps of reading the polypeptide code of SEQ ID NOS. 85-129 and 155-179 and the reference polypeptide sequence through use of a computer program which determines homology levels and determining homology between the polypeptide code and the reference polypeptide sequence using the computer program.

**[0257]** Accordingly, another aspect of the present invention is a method for determining whether a cDNA code of SEQ ID NOs. 40-84 and 130-154 differs at one or more nucleotides from a reference nucleotide sequence comprising the steps of reading the cDNA code and the reference nucleotide sequence through use of a computer program which identifies differences between nucleic acid sequences and identifying differences between the cDNA code and the reference nucleotide sequence with the computer program. In some embodiments, the computer program is a program which identifies single nucleotide polymorphisms. The method may be implemented by the computer systems described above. The method may also be performed by reading at least 2, 5, 10, 15, 20, 25, 30, or 50 of the cDNA codes of SEQ ID NOs. 40-84 and 130-154 and the reference nucleotide sequences through the use of the computer program and identifying differences between the cDNA codes and the reference nucleotide sequences with the computer program.

**[0258]** In other embodiments the computer based system may further comprise an identifier for identifying features within the nucleotide sequences of the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the amino acid sequences of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179.

**[0259]** An "identifier" refers to one or more programs which identifies certain features within the above-described nucleotide sequences of the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the amino acid sequences of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179. In one embodiment, the identifier may comprise a program which identifies an open reading frame in the cDNAs codes of SEQ ID NOs. 40-84 and 130-154.

**[0260]** In another embodiment, the identifier may comprise a molecular modeling program which determines the 3-dimensional structure of the polypeptides codes of SEQ ID NOS. 85-129 and 155-179. In some embodiments, the molecular modeling program identifies target sequences that are most compatible with profiles representing the structural environments of the residues in known three-dimensional protein structures. (See, e.g., Eisenberg et al., U.S. Patent No. 5,436,850 issued July 25, 1995). In another technique, the known three-dimensional structures of proteins in a given family are superimposed to define the structurally conserved regions in that family. This protein modeling technique also uses the known three-dimensional structure of a homologous protein to approximate the structure of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179. (See e.g., Srinivasan, et al., U.S. Patent No. 5,557,535 issued September 17, 1996). Conventional homology modeling techniques have been used routinely to build models of proteases and antibodies. (Sowdhamini et al., Protein Engineering 10:207, 215 (1997)). Comparative approaches can also be used to develop three-dimensional protein models when the protein of interest has poor sequence identity to template proteins. In some cases, proteins fold into similar three-dimensional structures despite having very weak sequence identities. For example, the three-dimensional structures of a number of helical cytokines fold in similar three-dimensional topology in spite of weak sequence homology.

**[0261]** The recent development of threading methods now enables the identification of likely folding patterns in a number of situations where the structural

relatedness between target and template(s) is not detectable at the sequence level. Hybrid methods, in which fold recognition is performed using Multiple Sequence Threading (MST), structural equivalencies are deduced from the threading output using a distance geometry program DRAGON to construct a low resolution model, and a full-atom representation is constructed using a molecular modeling package such as QUANTA.

**[0262]** According to this 3-step approach, candidate templates are first identified by using the novel fold recognition algorithm MST, which is capable of performing simultaneous threading of multiple aligned sequences onto one or more 3-D structures. In a second step, the structural equivalencies obtained from the MST output are converted into interresidue distance restraints and fed into the distance geometry program DRAGON, together with auxiliary information obtained from secondary structure predictions. The program combines the restraints in an unbiased manner and rapidly generates a large number of low resolution model confirmations. In a third step, these low resolution model confirmations are converted into full-atom models and subjected to energy minimization using the molecular modeling package QUANTA. (See e.g., Aszódi et al., Proteins:Structure, Function, and Genetics, Supplement 1:38-42 (1997)).

**[0263]** The results of the molecular modeling analysis may then be used in rational drug design techniques to identify agents which modulate the activity of the polypeptide codes of SEQ ID NOS. 85-129 and 155-179.

**[0264]** Accordingly, another aspect of the present invention is a method of identifying a feature within the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the polypeptide codes of SEQ ID NOS. 85-129 and 155-179 comprising reading the cDNA code(s) or the polypeptide code(s) through the use of a computer program which identifies features therein and identifying features within the cDNA code(s) or polypeptide code(s) with the computer program. In one embodiment, computer program comprises a computer program which identifies open reading frames. In a further embodiment, the computer program identifies structural motifs in a polypeptide sequence. In another embodiment, the computer program comprises a molecular modeling program. The method may be performed by reading a single sequence or at

least 2, 5, 10, 15, 20, 25, 30, or 50 of the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the polypeptide codes of SEQ ID NOS. 85-129 and 155-179 through the use of the computer program and identifying features within the cDNA codes or polypeptide codes with the computer program.

**[0265]** The cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the polypeptide codes of SEQ ID NOS. 85-129 and 155-179 may be stored and manipulated in a variety of data processor programs in a variety of formats. For example, the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the polypeptide codes of SEQ ID NOS. 85-129 and 155-179 may be stored as text in a word processing file, such as MicrosoftWORD or WORDPERFECT or as an ASCII file in a variety of database programs familiar to those of skill in the art, such as DB2, SYBASE, or ORACLE. In addition, many computer programs and databases may be used as sequence comparers, identifiers, or sources of reference nucleotide or polypeptide sequences to be compared to the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the polypeptide codes of SEQ ID NOS. 85-129 and 155-179. The following list is intended not to limit the invention but to provide guidance to programs and databases which are useful with the cDNA codes of SEQ ID NOs. 40-84 and 130-154 or the polypeptide codes of SEQ ID NOS. 85-129 and 155-179. The programs and databases which may be used include, but are not limited to: MacPattern (EMBL), DiscoveryBase (Molecular Applications Group), GeneMine (Molecular Applications Group), Look (Molecular Applications Group), MacLook (Molecular Applications Group), BLAST and BLAST2 (NCBI), BLASTN and BLASTX (Altschul et al, *J. Mol. Biol.* 215: 403 (1990)), FASTA (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA,* 85: 2444 (1988)), FASTDB (Brutlag et al. Comp. App. Biosci. 6:237-245, 1990), Catalyst (Molecular Simulations Inc.), Catalyst/SHAPE (Molecular Simulations Inc.), Cerius$^2$.DBAccess (Molecular Simulations Inc.), HypoGen (Molecular Simulations Inc.), Insight II, (Molecular Simulations Inc.), Discover (Molecular Simulations Inc.), CHARMm (Molecular Simulations Inc.), Felix (Molecular Simulations Inc.), DelPhi, (Molecular Simulations Inc.), QuanteMM, (Molecular Simulations Inc.), Homology (Molecular Simulations Inc.), Modeler (Molecular Simulations Inc.), ISIS (Molecular Simulations Inc.), Quanta/Protein Design (Molecular Simulations Inc.), WebLab (Molecular Simulations Inc.), WebLab Diversity Explorer (Molecular

Simulations Inc.), Gene Explorer (Molecular Simulations Inc.), SeqFold (Molecular Simulations Inc.), the EMBL/Swissprotein database, the MDL Available Chemicals Directory database, the MDL Drug Data Report data base, the Comprehensive Medicinal Chemistry database, Derwents's World Drug Index database, the BioByteMasterFile database, the Genbank database, and the Genseqn database. Many other programs and data bases would be apparent to one of skill in the art given the present disclosure.

**[0266]** Motifs which may be detected using the above programs include sequences encoding leucine zippers, helix-turn-helix motifs, glycosylation sites, ubiquitination sites, alpha helices, and beta sheets, signal sequences encoding signal peptides which direct the secretion of the encoded proteins, sequences implicated in transcription regulation such as homeoboxes, acidic stretches, enzymatic active sites, substrate binding sites, and enzymatic cleavage sites.

5. Selection of Cloned Full Length Sequences of the Present Invention

**[0267]** Cloned full length extended cDNA sequences that have already been characterized by the aforementioned computer analysis are then submitted to an automatic procedure in order to preselect full length extended cDNAs containing sequences of interest.

a) Automatic sequence preselection

**[0268]** All complete cloned full length extended cDNAs clipped for vector on both ends are considered. First, a negative selection is operated in order to eliminate unwanted cloned sequences resulting from either contaminants or PCR artifacts as follows. Sequences matching contaminant sequences such as vector RNA, tRNA, mtRNA, rRNA sequences are discarded as well as those encoding ORF sequences exhibiting extensive homology to repeats as defined in section 4 a). Sequences obtained by direct cloning using nested primers on 5' and 3' tags (section 1. case a) but lacking polyA tail are discarded. Only ORFs containing a signal peptide and ending either before the polyA tail (case a) or before the end of the cloned 3'UTR (case b) are kept. Then, ORFs containing unlikely mature proteins such as mature proteins which size is less than 20 amino acids or less than 25% of the immature protein size are eliminated.

**[0269]** In the selection of the ORF, priority was given to the ORF and the frame corresponding to the polypeptides described in SignalTag Patents (United States Patent Application Serial Nos: 08/905,223; 08/905,135; 08/905,051; 08/905,144; 08/905,279; 08/904,468; 08/905,134; and 08/905,133). If the ORF was not found among the ORFs described in the SignalTag Patents, the ORF encoding the signal peptide with the highest score according to Von Heijne method as defined in Example 22 was chosen. If the scores were identical, then the longest ORF was chosen.

**[0270]** Sequences of full length extended cDNA clones are then compared pairwise with BLAST after masking of the repeat sequences. Sequences containing at least 90% homology over 30 nucleotides are clustered in the same class. Each cluster is then subjected to a cluster analysis that detects sequences resulting from internal priming or from alternative splicing, identical sequences or sequences with several frameshifts. This automatic analysis serves as a basis for manual selection of the sequences.

b) Manual sequence selection

**[0271]** Manual selection can be carried out using automatically generated reports for each sequenced full length extended cDNA clone. During this manual procedure, a selection is operated between clones belonging to the same class as follows. ORF sequences encoded by clones belonging to the same class are aligned and compared. If the homology between nucleotidic sequences of clones belonging to the same class is more than 90% over 30 nucleotide stretches or if the homology between amino acid sequences of clones belonging to the same class is more than 80% over 20 amino acid stretches, than the clones are considered as being identical. The chosen ORF is the best one according to the criteria mentioned below. If the nucleotide and amino acid homologies are less than 90% and 80% respectively, the clones are said to encode distinct proteins which can be both selected if they contain sequences of interest.

**[0272]** Selection of full length extended cDNA clones encoding sequences of interest is performed using the following criteria. Structural parameters (initial tag, polyadenylation site and signal) are first checked. Then, homologies with known nucleic acids and proteins are examined in order to determine whether the clone sequence match a known nucleic/proteic sequence and, in the latter case, its covering rate and the date at which the sequence became public. If there is no extensive match with sequences other

than ESTs or genomic DNA, or if the clone sequence brings substantial new information, such as encoding a protein resulting from alternative slicing of an mRNA coding for an already known protein, the sequence is kept. Examples of such cloned full length extended cDNAs containing sequences of interest are described in Example 28. Sequences resulting from chimera or double inserts as assessed by homology to other sequences are discarded during this procedure.

## EXAMPLE 28

Cloning and Sequencing of Extended cDNAs

**[0273]** The procedure described in Example 27 above was used to obtain the extended cDNAs of the present invention. Using this approach, the full length cDNA of SEQ ID NO:17 was obtained. This cDNA falls into the "EST-ext" category described above and encodes the signal peptide MKKVLLLITAILAVAVG (SEQ ID NO: 18) having a von Heijne score of 8.2.

**[0274]** The full length cDNA of SEQ ID NO: 19 was also obtained using this procedure. This cDNA falls into the "EST-ext" category described above and encodes the signal peptide MWWFQQGLSFLPSALVIWTSA (SEQ ID NO:20) having a von Heijne score of 5.5.

**[0275]** Another full length cDNA obtained using the procedure described above has the sequence of SEQ ID NO:21. This cDNA, falls into the "EST-ext" category described above and encodes the signal peptide MVLTTLPSANSANSPVNMPTTGPNSLSYASSALSPCLT (SEQ ID NO:22) having a von Heijne score of 5.9.

**[0276]** The above procedure was also used to obtain a full length cDNA having the sequence of SEQ ID NO:23. This cDNA falls into the "EST-ext" category described above and encodes the signal peptide ILSTVTALTFAXA (SEQ ID NO:24) having a von Heijne score of 5.5.

**[0277]** The full length cDNA of SEQ ID NO:25 was also obtained using this procedure. This cDNA falls into the "new" category described above and encodes a signal peptide LVLTLCTLPLAVA (SEQ ID NO:26) having a von Heijne score of 10.1.

**[0278]** The full length cDNA of SEQ ID NO:27 was also obtained using this procedure. This cDNA falls into the "new" category described above and encodes a signal peptide LWLLFFLVTAIHA (SEQ ID NO:28) having a von Heijne score of 10.7.

**[0279]** The above procedures were also used to obtain the extended cDNAs of the present invention. 5′ ESTs expressed in a variety of tissues were obtained as described above. The appended sequence listing provides the tissues from which the extended cDNAs were obtained. It will be appreciated that the extended cDNAs may also be expressed in tissues other than the tissue listed in the sequence listing.

**[0280]** 5′ ESTs obtained as described above were used to obtain extended cDNAs having the sequences of SEQ ID NOs: 40-84 and 130-154. Table IV provides the sequence identification numbers of the extended cDNAs of the present invention, the locations of the full coding sequences in SEQ ID NOs: 40-84 and 130-154 (i.e. the nucleotides encoding both the signal peptide and the mature protein, listed under the heading FCS location in Table IV), the locations of the nucleotides in SEQ ID NOs: 40-84 and 130-154 which encode the signal peptides (listed under the heading SigPep Location in Table IV), the locations of the nucleotides in SEQ ID NOs: 40-84 and 130-154 which encode the mature proteins generated by cleavage of the signal peptides (listed under the heading Mature Polypeptide Location in Table IV), the locations in SEQ ID NOs: 40-84 and 130-154 of stop codons (listed under the heading Stop Codon Location in Table IV), the locations in SEQ ID NOs: 40-84 and 130-154 of polyA signals (listed under the heading Poly A Signal Location in Table IV) and the locations of polyA sites (listed under the heading Poly A Site Location in Table IV).

**[0281]** The polypeptides encoded by the extended cDNAs were screened for the presence of known structural or functional motifs or for the presence of signatures, small amino acid sequences which are well conserved amongst the members of a protein family. The conserved regions have been used to derive consensus patterns or matrices included in the PROSITE data bank, in particular in the file prosite.dat (Release 13.0 of

November 1995, located at http://expasy.hcuge.ch/sprot/prosite.html. Prosite_convert and prosite_scan programs (http://ulrec3.unil.ch/ftpserveur/prosite_scan) were used to find signatures on the extended cDNAs.

**[0282]** For each pattern obtained with the prosite_convert program from the prosite.dat file, the accuracy of the detection on a new protein sequence has been tested by evaluating the frequency of irrelevant hits on the population of human secreted proteins included in the data bank SWISSPROT. The ratio between the number of hits on shuffled proteins (with a window size of 20 amino acids) and the number of hits on native (unshuffled) proteins was used as an index. Every pattern for which the ration was greater than 20% (one hit on shuffled proteins for 5 hits on native proteins) was skipped during the search with prosite_scan. The program used to shuffle protein sequences (db_shuffled) and the program used to determine the statistics for each pattern in the protein data banks (prosite_statistics) are available on the ftp site http://ulrec3.unil.ch/ftpserveur/prosite_scan.

**[0283]** Table V lists the sequence identification numbers of the polypeptides of SEQ ID NOs: 85-129 and 155-179, the locations of the amino acid residues of SEQ ID NOs: 85-129 and 155-179 in the full length polypeptide (second column), the locations of the amino acid residues of SEQ ID NOs: 85-129 and 155-179 in the signal peptides (third column), and the locations of the amino acid residues of SEQ ID NOs: 85-129 and 155-179 in the mature polypeptide created by cleaving the signal peptide from the full length polypeptide (fourth column).

**[0284]** The nucleotide sequences of the sequences of SEQ ID NOs: 40-84 and 130-154 and the amino acid sequences encoded by SEQ ID NOs: 40-84 and 130-154 (i.e. amino acid sequences of SEQ ID NOs: 85-129 and 155-179) are provided in the appended sequence listing. In some instances, the sequences are preliminary and may include some incorrect or ambiguous sequences or amino acids. The sequences of SEQ ID NOs: 40-84 and 130-154 can readily be screened for any errors therein and any sequence ambiguities can be resolved by resequencing a fragment containing such errors or ambiguities on both strands. Sequences containing such errors will generally be at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% homologous to the sequences of SEQ ID Nos. 85-129 and 155-179 and such sequences are included in the nucleic acids and polypeptides of the present invention. Nucleic acid fragments for resolving

sequencing errors or ambiguities may be obtained from the deposited clones or can be isolated using the techniques described herein. Resolution of any such ambiguities or errors may be facilitated by using primers which hybridize to sequences located close to the ambiguous or erroneous sequences. For example, the primers may hybridize to sequences within 50-75 bases of the ambiguity or error. Upon resolution of an error or ambiguity, the corresponding corrections can be made in the protein sequences encoded by the DNA containing the error or ambiguity. The amino acid sequence of the protein encoded by a particular clone can also be determined by expression of the clone in a suitable host cell, collecting the protein, and determining its sequence.

**[0285]** For each amino acid sequence, Applicants have identified what they have determined to be the reading frame best identifiable with sequence information available at the time of filing. Some of the amino acid sequences may contain "Xaa" designators. These "Xaa" designators indicate either (1) a residue which cannot be identified because of nucleotide sequence ambiguity or (2) a stop codon in the determined sequence where Applicants believe one should not exist (if the sequence were determined more accurately).

**[0286]** Cells containing the extended cDNAs (SEQ ID NOs: 40-84 and 130-154) of the present invention in the vector pED6dpc2, are maintained in permanent deposit by the inventors at Genset, S.A., 24 Rue Royale, 75008 Paris, France.

**[0287]** Pools of cells containing the extended cDNAs (SEQ ID NOs: 40-84), from which cells containing a particular polynucleotide are obtainable, were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA, U.S.A., 20110-2209. Each extended cDNA clone has been transfected into separate bacterial cells (E-coli) for this composite deposit. Table VI lists the deposit numbers of the clones of SEQ ID Nos: 40-84. A pool of cells designated SignalTag 28011999, which contains the clones of SEQ ID NOs 71-84 was mailed to the European Collection of Cell Cultures, (ECACC) Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom on January 28, 1999 and was received on January 29, 1999. This pool of cells has the ECACC Accession # XXXXXX. One or more pools of cells containing the extended cDNAs of SEQ ID Nos: 130-154, from

which the cells containing a particular polynucleotide is obtainable, will be deposited with the European Collection of Cell Cultures, Vaccine Research and Production Laboratory, Public Health Laboratory Service, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom and will be assigned ECACC deposit number XXXXXXX. Table VII provides the internal designation number assigned to each SEQ ID NO. and indicates whether the sequence is a nucleic acid sequence or a protein sequence.

**[0288]** Each extended cDNA can be removed from the pED6dpc2 vector in which it was deposited by performing a NotI, PstI double digestion to produce the appropriate fragment for each clone. The proteins encoded by the extended cDNAs may also be expressed from the promoter in pED6dpc2.

**[0289]** Bacterial cells containing a particular clone can be obtained from the composite deposit as follows:

**[0290]** An oligonucleotide probe or probes should be designed to the sequence that is known for that particular clone. This sequence can be derived from the sequences provided herein, or from a combination of those sequences. The design of the oligonucleotide probe should preferably follow these parameters:

(a) It should be designed to an area of the sequence which has the fewest ambiguous bases ("N's"), if any;

(b) Preferably, the probe is designed to have a $T_m$ of approx. 80°C (assuming 2 degrees for each A or T and 4 degrees for each G or C). However, probes having melting temperatures between 40 °C and 80 °C may also be used provided that specificity is not lost.

**[0291]** The oligonucleotide should preferably be labeled with (-[$^{32}$P]ATP (specific activity 6000 Ci/mmole) and T4 polynucleotide kinase using commonly employed techniques for labeling oligonucleotides. Other labeling techniques can also be used. Unincorporated label should preferably be removed by gel filtration chromatography or other established methods. The amount of radioactivity incorporated into the probe should be quantified by measurement in a scintillation counter. Preferably, specific activity of the resulting probe should be approximately 4X10$^6$ dpm/pmole.

**[0292]** The bacterial culture containing the pool of full-length clones should preferably be thawed and 100 µl of the stock used to inoculate a sterile culture flask containing 25 ml of sterile L-broth containing ampicillin at 100 ug/ml. The culture should preferably be grown to saturation at 37°C, and the saturated culture should preferably be diluted in fresh L-broth. Aliquots of these dilutions should preferably be plated to determine the dilution and volume which will yield approximately 5000 distinct and well-separated colonies on solid bacteriological media containing L-broth containing ampicillin at 100 µg/ml and agar at 1.5% in a 150 mm petri dish when grown overnight at 37°C. Other known methods of obtaining distinct, well-separated colonies can also be employed.

**[0293]** Standard colony hybridization procedures should then be used to transfer the colonies to nitrocellulose filters and lyse, denature and bake them.

**[0294]** The filter is then preferably incubated at 65°C for 1 hour with gentle agitation in 6X SSC (20X stock is 175.3 g NaCl/liter, 88.2 g Na citrate/liter, adjusted to pH 7.0 with NaOH) containing 0.5% SDS, 100 pg/ml of yeast RNA, and 10 mM EDTA (approximately 10 mL per 150 mm filter). Preferably, the probe is then added to the hybridization mix at a concentration greater than or equal to $1X10^6$ dpm/mL. The filter is then preferably incubated at 65°C with gentle agitation overnight. The filter is then preferably washed in 500 mL of 2X SSC/0.1% SDS at room temperature with gentle shaking for 15 minutes. A third wash with 0.1X SSC/0.5% SDS at 65°C for 30 minutes to 1 hour is optional. The filter is then preferably dried and subjected to autoradiography for sufficient time to visualize the positives on the X-ray film. Other known hybridization methods can also be employed.

**[0295]** The positive colonies are picked, grown in culture, and plasmid DNA isolated using standard procedures. The clones can then be verified by restriction analysis, hybridization analysis, or DNA sequencing.

**[0296]** The plasmid DNA obtained using these procedures may then be manipulated using standard cloning techniques familiar to those skilled in the art. Alternatively, a PCR can be done with primers designed at both ends of the extended cDNA insertion. For example, a PCR reaction may be conducted using a primer having the sequence GGCCATACACTTGAGTGAC (SEQ ID NO:38) and a primer having the

sequence ATATAGACAAACGCACACC (SEQ. ID. NO:39). The PCR product which corresponds to the extended cDNA can then be manipulated using standard cloning techniques familiar to those skilled in the art.

**[0297]** In addition to PCR based methods for obtaining extended cDNAs, traditional hybridization based methods may also be employed. These methods may also be used to obtain the genomic DNAs which encode the mRNAs from which the 5′ ESTs were derived, mRNAs corresponding to the extended cDNAs, or nucleic acids which are homologous to extended cDNAs or 5′ ESTs. Example 29 below provides an example of such methods.

## EXAMPLE 29

Methods for Obtaining Extended cDNAs or Nucleic

Acids Homologous to Extended cDNAs or 5′ ESTs

**[0298]** 5'ESTs or extended cDNAs of the present invention may also be used to isolate extended cDNAs or nucleic acids homologous to extended cDNAs from a cDNA library or a genomic DNA library. Such cDNA library or genomic DNA library may be obtained from a commercial source or made using other techniques familiar to those skilled in the art. One example of such cDNA library construction is as follows.

**[0299]** PolyA+ RNAs are prepared and their quality checked as described in Example 13. Then, polyA+ RNAs are ligated to an oligonucleotide tag using either the chemical or enzymatic methods described in above sections 1 and 2. In both cases, the oligonucleotide tag may contain a restriction site such as Eco RI to facilitate further subcloning procedures. Northern blotting is then performed to check the size of ligatured mRNAs and to ensure that the mRNAs were actually tagged.

**[0300]** As described in Example 14, first strand synthesis is subsequently carried out for mRNAs joined to the oligonucleotide tag replacing the random nonamers by an oligodT primer. For instance, this oligodT primer may contain an internal tag of 4 nucleotides which is different from one tissue to the other. Alternatively, the oligonucleotide of SEQ ID NO:14 may be used. Following second strand synthesis using

a primer contained in the oligonucleotide tag attached to the 5' end of mRNA, the blunt ends of the obtained double stranded full length DNAs are modified into cohesive ends to allow subcloning into the Eco RI and Hind III sites of a Bluescript vector using the addition of a Hind III adaptor to the 3' end of full length DNAs.

**[0301]** The extended full length DNAs are then separated into several fractions according to their sizes using techniques familiar to those skilled in the art. For example, electrophoretic separation may be applied in order to yield 3 or 6 different fractions. Following gel extraction and purification, the DNA fractions are subcloned into Bluescript vectors, transformed into competent bacteria and propagated under appropriate antibiotic conditions.

**[0302]** Such full length cDNA libraries may then be sequenced as follows or used in screening procedures to obtain nucleic acids homologous to extended cDNAs or 5' ESTs as described below.

**[0303]** The 5' end of extended cDNA isolated from the full length cDNA libraries or of nucleic acid homologous thereto may then be sequenced as described in example 27. In a first step, the sequence corresponding to the 5' end of the mRNA is obtained. If this sequence either corresponds to a SignalTag™ 5'EST or fulfills the criteria to be one, the cloned insert is subcloned into an appropriate vector such as pED6dpc2, double-sequenced and submitted to the analysis and selection procedures described in Example 27.

**[0304]** Such cDNA or genomic DNA libraries may be used to isolate extended cDNAs obtained from 5′ EST or nucleic acids homologous to extended cDNAs or 5′ EST as follows. The cDNA library or genomic DNA library is hybridized to a detectable probe comprising at least 10 consecutive nucleotides from the 5′ EST or extended cDNA using conventional techniques. Preferably, the probe comprises at least 12, 15, or 17 consecutive nucleotides from the 5′ EST or extended cDNA. More preferably, the probe comprises at least 20 to 30 consecutive nucleotides from the 5′ EST or extended cDNA. In some embodiments, the probe comprises at least 40, at least 50, at least 75, at least 100, at least 150, or at least 200 consecutive nucleotides from the 5′ EST or extended cDNA. **[0302]** Techniques for identifying cDNA clones in a cDNA library

which hybridize to a given probe sequence are disclosed in Sambrook *et al., Molecular Cloning: A Laboratory Manual 2d Ed.*, Cold Spring Harbor Laboratory Press, 1989, the disclosure of which is incorporated herein by reference. The same techniques may be used to isolate genomic DNAs.

**[0305]** Briefly, cDNA or genomic DNA clones which hybridize to the detectable probe are identified and isolated for further manipulation as follows. A probe comprising at least 10 consecutive nucleotides from the 5′ EST or extended cDNA is labeled with a detectable label such as a radioisotope or a fluorescent molecule. Preferably, the probe comprises at least 12, 15, or 17 consecutive nucleotides from the 5′ EST or extended cDNA. More preferably, the probe comprises 20 to 30 consecutive nucleotides from the 5′ EST or extended cDNA. In some embodiments, the probe comprises at least 40, at least 50, at least 75, at least 100, at least 150, or at least 200 consecutive nucleotides from the 5′ EST or extended cDNA. **[0304]** Techniques for labeling the probe are well known and include phosphorylation with polynucleotide kinase, nick translation, *in vitro* transcription, and non-radioactive techniques. The cDNAs or genomic DNAs in the library are transferred to a nitrocellulose or nylon filter and denatured. After blocking of non-specific sites, the filter is incubated with the labeled probe for an amount of time sufficient to allow binding of the probe to cDNAs or genomic DNAs containing a sequence capable of hybridizing thereto.

**[0306]** By varying the stringency of the hybridization conditions used to identify extended cDNAs or genomic DNAs which hybridize to the detectable probe, extended cDNAS having different levels of homology to the probe can be identified and isolated as described below.

1. Identification of Extended cDNA or Genomic DNA Sequences Having a High Degree of Homology to the Labeled Probe

**[0307]** To identify extended cDNAs or genomic DNAs having a high degree of homology to the probe sequence, the melting temperature of the probe may be calculated using the following formulas:

**[0308]** For probes between 14 and 70 nucleotides in length the melting temperature (Tm) is calculated using the formula: Tm=81.5+16.6(log [Na+])+0.41(fraction G+C)-(600/N) where N is the length of the probe.

**[0309]** If the hybridization is carried out in a solution containing formamide, the melting temperature may be calculated using the equation Tm=81.5+16.6(log [Na+])+0.41(fraction G+C)-(0.63% formamide)-(600/N) where N is the length of the probe.

**[0310]** Prehybridization may be carried out in 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 μg denatured fragmented salmon sperm DNA or 6X SSC, 5X Denhardt's reagent, 0.5% SDS, 100 μg denatured fragmented salmon sperm DNA, 50% formamide. The formulas for SSC and Denhardt's solutions are listed in Sambrook *et al.*, *supra*.

**[0311]** Hybridization is conducted by adding the detectable probe to the prehybridization solutions listed above. Where the probe comprises double stranded DNA, it is denatured before addition to the hybridization solution. The filter is contacted with the hybridization solution for a sufficient period of time to allow the probe to hybridize to extended cDNAs or genomic DNAs containing sequences complementary thereto or homologous thereto. For probes over 200 nucleotides in length, the hybridization may be carried out at 15-25°C below the Tm. For shorter probes, such as oligonucleotide probes, the hybridization may be conducted at 15-25°C below the Tm. Preferably, for hybridizations in 6X SSC, the hybridization is conducted at approximately 68°C. Preferably, for hybridizations in 50% formamide containing solutions, the hybridization is conducted at approximately 42°C.

**[0312]** All of the foregoing hybridizations would be considered to be under "stringent" conditions.

**[0313]** Following hybridization, the filter is washed in 2X SSC, 0.1% SDS at room temperature for 15 minutes. The filter is then washed with 0.1X SSC, 0.5% SDS at room temperature for 30 minutes to 1 hour. Thereafter, the solution is washed at the hybridization temperature in 0.1X SSC, 0.5% SDS. A final wash is conducted in 0.1X SSC at room temperature.

**[0314]** Extended cDNAs, nucleic acids homologous to extended cDNAs or 5′ ESTs, or genomic DNAs which have hybridized to the probe are identified by autoradiography or other conventional techniques.

2. Obtaining Extended cDNA or Genomic DNA Sequences Having Lower Degrees of Homology to the Labeled Probe

**[0315]** The above procedure may be modified to identify extended cDNAs, nucleic acids homologous to extended cDNAs, or genomic DNAs having decreasing levels of homology to the probe sequence. For example, to obtain extended cDNAs, nucleic acids homologous to extended cDNAs, or genomic DNAs of decreasing homology to the detectable probe, less stringent conditions may be used. For example, the hybridization temperature may be decreased in increments of 5°C from 68°C to 42°C in a hybridization buffer having a sodium concentration of approximately 1M. Following hybridization, the filter may be washed with 2X SSC, 0.5% SDS at the temperature of hybridization. These conditions are considered to be "moderate" conditions above 50°C and "low" conditions below 50°C.

**[0316]** Alternatively, the hybridization may be carried out in buffers, such as 6X SSC, containing formamide at a temperature of 42°C. In this case, the concentration of formamide in the hybridization buffer may be reduced in 5% increments from 50% to 0% to identify clones having decreasing levels of homology to the probe. Following hybridization, the filter may be washed with 6X SSC, 0.5% SDS at 50°C. These conditions are considered to be "moderate" conditions above 25% formamide and "low" conditions below 25% formamide.

**[0317]** Extended cDNAs, nucleic acids homologous to extended cDNAs, or genomic DNAs which have hybridized to the probe are identified by autoradiography.

3. Determination of the Degree of Homology between the Obtained Extended cDNAs or Genomic DNAs and the Labeled Probe

**[0318]** To determine the level of homology between the hybridized nucleic acid and the extended cDNA or 5′EST from which the probe was derived, the nucleotide

sequences of the hybridized nucleic acid and the extended cDNA or 5'EST from which the probe was derived are compared. The sequences of the extended cDNA or 5'EST and the homologous sequences may be stored on a computer readable medium as described in Example 17 above and may be compared using any of a variety of algorithms familiar to those skilled in the art. For example, if it is desired to obtain nucleic acids homologous to extended cDNAs, such as allelic variants thereof or nucleic acids encoding proteins related to the proteins encoded by the extended cDNAs, the level of homology between the hybridized nucleic acid and the extended cDNA or 5' EST used as the probe may be determined using algorithms such as BLAST2N; parameters may be adapted depending on the sequence length and degree of homology studied. For example, the default parameters or the parameters in Table I and II may be used to determine homology levels.

**[0319]** Alternatively, the level of homology between the hybridized nucleic acid and the extended cDNA or 5'EST from which the probe was derived may be determined using the FASTDB algorithm described in Brutlag et al. Comp. App. Biosci. 6:237-245, 1990. In such analyses the parameters may be selected as follows: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the sequence which hybridizes to the probe, whichever is shorter. Because the FASTDB program does not consider 5' or 3' truncations when calculating homology levels, if the sequence which hybridizes to the probe is truncated relative to the sequence of the extended cDNA or 5'EST from which the probe was derived the homology level is manually adjusted by calculating the number of nucleotides of the extended cDNA or 5'EST which are not matched or aligned with the hybridizing sequence, determining the percentage of total nucleotides of the hybridizing sequence which the non-matched or non-aligned nucleotides represent, and subtracting this percentage from the homology level. For example, if the hybridizing sequence is 700 nucleotides in length and the extended cDNA sequence is 1000 nucleotides in length wherein the first 300 bases at the 5' end of the extended cDNA are absent from the hybridizing sequence, and wherein the overlapping 700 nucleotides are identical, the homology level would be adjusted as follows. The non-matched, non-aligned 300 bases represent 30% of the length of the extended cDNA. If the overlapping 700 nucleotides are 100% identical, the adjusted

homology level would be 100-30=70% homology. It should be noted that the preceding adjustments are only made when the non-matched or non-aligned nucleotides are at the 5′ or 3′ ends. No adjustments are made if the non-matched or non-aligned sequences are internal or under any other conditions.

**[0320]** For example, using the above methods, nucleic acids having at least 95% nucleic acid homology, at least 96% nucleic acid homology, at least 97% nucleic acid homology, at least 98% nucleic acid homology, at least 99% nucleic acid homology, or more than 99% nucleic acid homology to the extended cDNA or 5′EST from which the probe was derived may be obtained and identified. Such nucleic acids may be allelic variants or related nucleic acids from other species. Similarly, by using progressively less stringent hybridization conditions one can obtain and identify nucleic acids having at least 90%, at least 85%, at least 80% or at least 75% homology to the extended cDNA or 5′EST from which the probe was derived.

**[0321]** To determine whether a clone encodes a protein having a given amount of homology to the protein encoded by the extended cDNA or 5′ EST, the amino acid sequence encoded by the extended cDNA or 5′ EST is compared to the amino acid sequence encoded by the hybridizing nucleic acid. The sequences encoded by the extended cDNA or 5′EST and the sequences encoded by the homologous sequences may be stored on a computer readable medium as described in Example 17 above and may be compared using any of a variety of algorithms familiar to those skilled in the art. Homology is determined to exist when an amino acid sequence in the extended cDNA or 5′ EST is closely related to an amino acid sequence in the hybridizing nucleic acid. A sequence is closely related when it is identical to that of the extended cDNA or 5′ EST or when it contains one or more amino acid substitutions therein in which amino acids having similar characteristics have been substituted for one another. Using the above methods and algorithms such as FASTA with parameters depending on the sequence length and degree of homology studied, for example the default parameters or the parameters in Table I and II, one can obtain nucleic acids encoding proteins having at least 99%, at least 98%, at least 97%, at least 96%, at least 95%, at least 90%, at least 85%, at least 80% or at least 75% homology to the proteins encoded by the extended cDNA or 5′EST from which the probe was derived. In some embodiments, the homology levels can be determined using

the "default" opening penalty and the "default" gap penalty, and a scoring matrix such as PAM 250 (a standard scoring matrix; see Dayhoff et al., in: Atlas of Protein Sequence and Structure, Vol. 5, Supp. 3 (1978)).

**[0322]** Alternatively, the level of homology may be determined using the FASTDB algorithm described by Brutlag et al. Comp. App. Biosci. 6:237-245, 1990. In such analyses the parameters may be selected as follows: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=Sequence Length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the homologous sequence, whichever is shorter. If the homologous amino acid sequence is shorter than the amino acid sequence encoded by the extended cDNA or 5'EST as a result of an N terminal and/or C terminal deletion the results may be manually corrected as follows. First, the number of amino acid residues of the amino acid sequence encoded by the extended cDNA or 5'EST which are not matched or aligned with the homologous sequence is determined. Then, the percentage of the length of the sequence encoded by the extended cDNA or 5'EST which the non-matched or non-aligned amino acids represent is calculated. This percentage is subtracted from the homology level. For example wherein the amino acid sequence encoded by the extended cDNA or 5'EST is 100 amino acids in length and the length of the homologous sequence is 80 amino acids and wherein the amino acid sequence encoded by the extended cDNA or 5'EST is truncated at the N terminal end with respect to the homologous sequence, the homology level is calculated as follows. In the preceding scenario there are 20 non-matched, non-aligned amino acids in the sequence encoded by the extended cDNA or 5'EST. This represents 20% of the length of the amino acid sequence encoded by the extended cDNA or 5'EST. If the remaining amino acids are 1005 identical between the two sequences, the homology level would be 100%-20%=80% homology. No adjustments are made if the non-matched or non-aligned sequences are internal or under any other conditions.

**[0323]** In addition to the above described methods, other protocols are available to obtain extended cDNAs using 5' ESTs as outlined in the following paragraphs.

**[0324]** Extended cDNAs may be prepared by obtaining mRNA from the tissue, cell, or organism of interest using mRNA preparation procedures utilizing polyA selection procedures or other techniques known to those skilled in the art. A first primer capable of hybridizing to the polyA tail of the mRNA is hybridized to the mRNA and a reverse transcription reaction is performed to generate a first cDNA strand.

**[0325]** The first cDNA strand is hybridized to a second primer containing at least 10 consecutive nucleotides of the sequences of the 5′ EST for which an extended cDNA is desired. Preferably, the primer comprises at least 12, 15, or 17 consecutive nucleotides from the sequences of the 5′ EST. More preferably, the primer comprises 20 to 30 consecutive nucleotides from the sequences of the 5′ EST. In some embodiments, the primer comprises more than 30 nucleotides from the sequences of the 5′ EST. If it is desired to obtain extended cDNAs containing the full protein coding sequence, including the authentic translation initiation site, the second primer used contains sequences located upstream of the translation initiation site. The second primer is extended to generate a second cDNA strand complementary to the first cDNA strand. Alternatively, RT-PCR may be performed as described above using primers from both ends of the cDNA to be obtained.

**[0326]** Extended cDNAs containing 5′ fragments of the mRNA may be prepared by hybridizing an mRNA comprising the sequence of the 5′ EST for which an extended cDNA is desired with a primer comprising at least 10 consecutive nucleotides of the sequences complementary to the 5′ EST and reverse transcribing the hybridized primer to make a first cDNA strand from the mRNAs. Preferably, the primer comprises at least 12, 15, or 17 consecutive nucleotides from the 5′ EST. More preferably, the primer comprises 20 to 30 consecutive nucleotides from the 5′ EST.

**[0327]** Thereafter, a second cDNA strand complementary to the first cDNA strand is synthesized. The second cDNA strand may be made by hybridizing a primer complementary to sequences in the first cDNA strand to the first cDNA strand and extending the primer to generate the second cDNA strand.

**[0328]** The double stranded extended cDNAs made using the methods described above are isolated and cloned. The extended cDNAs may be cloned into vectors

such as plasmids or viral vectors capable of replicating in an appropriate host cell. For example, the host cell may be a bacterial, mammalian, avian, or insect cell.

**[0329]** Techniques for isolating mRNA, reverse transcribing a primer hybridized to mRNA to generate a first cDNA strand, extending a primer to make a second cDNA strand complementary to the first cDNA strand, isolating the double stranded cDNA and cloning the double stranded cDNA are well known to those skilled in the art and are described *in Current Protocols in Molecular Biology*, John Wiley 503 Sons, Inc. 1997 and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989, the entire disclosures of which are incorporated herein by reference.

**[0330]** Alternatively, other procedures may be used for obtaining full length cDNAs or extended cDNAs. In one approach, full length or extended cDNAs are prepared from mRNA and cloned into double stranded phagemids as follows. The cDNA library in the double stranded phagemids is then rendered single stranded by treatment with an endonuclease, such as the Gene II product of the phage F1, and an exonuclease (Chang *et al.*, *Gene* 127:95-8, 1993). A biotinylated oligonucleotide comprising the sequence of a 5′ EST, or a fragment containing at least 10 nucleotides thereof, is hybridized to the single stranded phagemids. Preferably, the fragment comprises at least 12, 15, or 17 consecutive nucleotides from the 5′ EST. More preferably, the fragment comprises 20-30 consecutive nucleotides from the 5′ EST. In some procedures, the fragment may comprise at least 40, at least 50, at least 75, at least 100, at least 150, or at least 200 conscutive nucleotides from the 5′ EST.

**[0331]** Hybrids between the biotinylated oligonucleotide and phagemids having inserts containing the 5′ EST sequence are isolated by incubating the hybrids with streptavidin coated paramagnetic beads and retrieving the beads with a magnet (Fry *et al.*, *Biotechniques*, 13: 124-131, 1992). Thereafter, the resulting phagemids containing the 5′ EST sequence are released from the beads and converted into double stranded DNA using a primer specific for the 5′ EST sequence. Alternatively, protocols such as the Gene Trapper kit (Gibco BRL) may be used. The resulting double stranded DNA is transformed

into bacteria. Extended cDNAs containing the 5′ EST sequence are identified by colony PCR or colony hybridization.

**[0332]** Using any of the above described methods in section III, a plurality of extended cDNAs containing full length protein coding sequences or sequences encoding only the mature protein remaining after the signal peptide is cleaved off may be provided as cDNA libraries for subsequent evaluation of the encoded proteins or use in diagnostic assays as described below.

## IV. Expression of Proteins Encoded by Extended cDNAs Isolated Using 5′ ESTs

**[0333]** Extended cDNAs containing the full protein coding sequences of their corresponding mRNAs or portions thereof, such as cDNAs encoding the mature protein, may be used to express the secreted proteins or portions thereof which they encode as described in Example 30 below. If desired, the extended cDNAs may contain the sequences encoding the signal peptide to facilitate secretion of the expressed protein. It will be appreciated that a plurality of extended cDNAs containing the full protein coding sequences or portions thereof may be simultaneously cloned into expression vectors to create an expression library for analysis of the encoded proteins as described below.

## EXAMPLE 30

Expression of the Proteins Encoded by Extended cDNAs or Portions Thereof

**[0334]** To express the proteins encoded by the extended cDNAs or portions thereof, nucleic acids containing the coding sequence for the proteins or portions thereof to be expressed are obtained as described in Examples 27-29 and cloned into a suitable expression vector. If desired, the nucleic acids may contain the sequences encoding the signal peptide to facilitate secretion of the expressed protein. For example, the nucleic acid may comprise the sequence of one of SEQ ID NOs: 40-84 and 130-154 listed in Table IV and in the accompanying sequence listing. Alternatively, the nucleic acid may comprise those nucleotides which make up the full coding sequence of one of the sequences of SEQ ID NOs: 40-84 and 130-154 as defined in Table IV above.

**[0335]** It will be appreciated that should the extent of the full coding sequence (i.e. the sequence encoding the signal peptide and the mature protein resulting from cleavage of the signal peptide) differ from that listed in Table IV as a result of a sequencing error, reverse transcription or amplification error, mRNA splicing, post-translational modification of the encoded protein, enzymatic cleavage of the encoded protein, or other biological factors, one skilled in the art would be readily able to identify the extent of the full coding sequences in the sequences of SEQ ID NOs. 40-84 and 130-154. Accordingly, the scope of any claims herein relating to nucleic acids containing the full coding sequence of one of SEQ ID NOs. 40-84 and 130-154 is not to be construed as excluding any readily identifiable variations from or equivalents to the full coding sequences listed in Table IV Similarly, should the extent of the full length polypeptides differ from those indicated in Table V as a result of any of the preceding factors, the scope of claims relating to polypeptides comprising the amino acid sequence of the full length polypeptides is not to be construed as excluding any readily identifiable variations from or equivalents to the sequences listed in Table V.

**[0336]** Alternatively, the nucleic acid used to express the protein or portion thereof may comprise those nucleotides which encode the mature protein (i.e. the protein created by cleaving the signal peptide off) encoded by one of the sequences of SEQ ID NOs: 40-84 and 130-154 as defined in Table IV above.

**[0337]** It will be appreciated that should the extent of the sequence encoding the mature protein differ from that listed in Table IV as a result of a sequencing error, reverse transcription or amplification error, mRNA splicing, post-translational modification of the encoded protein, enzymatic cleavage of the encoded protein, or other biological factors, one skilled in the art would be readily able to identify the extent of the sequence encoding the mature protein in the sequences of SEQ ID NOs. 40-84 and 130-154. Accordingly, the scope of any claims herein relating to nucleic acids containing the sequence encoding the mature protein encoded by one of SEQ ID Nos. 40-84 and 130-154 is not to be construed as excluding any readily identifiable variations from or equivalents to the sequences listed in Table IV. Thus, claims relating to nucleic acids containing the sequence encoding the mature protein encompass equivalents to the sequences listed in Table IV, such as sequences encoding biologically active proteins resulting from post-translational modification, enzymatic cleavage, or other readily identifiable variations from or equivalents to the secreted proteins in addition to cleavage of the signal peptide. Similarly, should the extent of the mature polypeptides differ from those indicated in Table V as a result of any of the preceding factors, the scope of claims relating to polypeptides comprising the sequence of a mature protein included in the sequence of one of SEQ ID NOs. 85-129 and 155-179 is not to be construed as excluding any readily identifiable variations from or equivalents to the sequences listed in Table V. Thus, claims relating to polypeptides comprising the sequence of the mature protein encompass equivalents to the sequences listed in Table IV, such as biologically active proteins resulting from post-translational modification, enzymatic cleavage, or other readily identifiable variations from or equivalents to the secreted proteins in addition to cleavage of the signal peptide. It will also be appreciated that should the biologically active form of the polypeptides included in the sequence of one of SEQ ID NOs. 85-129 and 155-179 or the nucleic acids encoding the biologically active form of the polypeptides differ from those identified as the mature polypeptide in Table V or the nucleotides encoding the mature polypeptide in Table IV as a result of a sequencing error, reverse transcription or amplification error, mRNA splicing, post-translational modification of the encoded protein, enzymatic cleavage of the encoded protein, or other biological factors, one skilled in the art would be readily able to identify the amino acids in the biologically active form of the polypeptides and the nucleic acids encoding the biologically active form of the polypeptides. In such instances, the claims

relating to polypetides comprising the mature protein included in one of SEQ ID NOs. 85-129 and 155-179 or nucleic acids comprising the nucleotides of one of SEQ ID NOs. 40-84 and 130-154 encoding the mature protein shall not be construed to exclude any readily identifiable variations from the sequences listed in Table IV and Table V.

**[0338]** In some embodiments, the nucleic acid used to express the protein or portion thereof may comprise those nucleotides which encode the signal peptide encoded by one of the sequences of SEQ ID NOs: 40-84 and 130-154 as defined in Table IV above.

**[0339]** It will be appreciated that should the extent of the sequence encoding the signal peptide differ from that listed in Table IV as a result of a sequencing error, reverse transcription or amplification error, mRNA splicing, post-translational modification of the encoded protein, enzymatic cleavage of the encoded protein, or other biological factors, one skilled in the art would be readily able to identify the extent of the sequence encoding the signal peptide in the sequences of SEQ ID NOs. 40-84 and 130-154. Accordingly, the scope of any claims herein relating to nucleic acids containing the sequence encoding the signal peptide encoded by one of SEQ ID Nos. 40-84 and 130-154 is not to be construed as excluding any readily identifiable variations from the sequences listed in Table IV. Similarly, should the extent of the signal peptides differ from those indicated in Table V as a result of any of the preceding factors, the scope of claims relating to polypeptides comprising the sequence of a signal peptide included in the sequence of one of SEQ ID NOs. 85-129 and 155-179 is not to be construed as excluding any readily identifiable variations from the sequences listed in Table V.

**[0340]** Alternatively, the nucleic acid may encode a polypeptide comprising at least 10 consecutive amino acids of one of the sequences of SEQ ID NOs: 85-129 and 155-179. In some embodiments, the nucleic acid may encode a polypeptide comprising at least 15 consecutive amino acids of one of the sequences of SEQ ID NOs: 85-129 and 155-179. In other embodiments, the nucleic acid may encode a polypeptide comprising at least 25 consecutive amino acids of one of the sequences of SEQ ID NOs: 85-129 and 155-179. In other embodiments, the nucleic acid may encode a polypeptide comprising at least 60, at least 75, at least 100 or more than 100 consecutive amino acids of one of the sequences of SEQ ID Nos: 85-129 and 155-179.

**[0341]** The nucleic acids inserted into the expression vectors may also contain sequences upstream of the sequences encoding the signal peptide, such as sequences which regulate expression levels or sequences which confer tissue specific expression.

**[0342]** The nucleic acid encoding the protein or polypeptide to be expressed is operably linked to a promoter in an expression vector using conventional cloning technology. The expression vector may be any of the mammalian, yeast, insect or bacterial expression systems known in the art. Commercially available vectors and expression systems are available from a variety of suppliers including Genetics Institute (Cambridge, MA), Stratagene (La Jolla, California), Promega (Madison, Wisconsin), and Invitrogen (San Diego, California). If desired, to enhance expression and facilitate proper protein folding, the codon context and codon pairing of the sequence may be optimized for the particular expression organism in which the expression vector is introduced, as explained by Hatfield, et al., U.S. Patent No. 5,082,767, incorporated herein by this reference.

**[0343]** The following is provided as one exemplary method to express the proteins encoded by the extended cDNAs corresponding to the 5′ ESTs or the nucleic acids described above. First, the methionine initiation codon for the gene and the poly A signal of the gene are identified. If the nucleic acid encoding the polypeptide to be expressed lacks a methionine to serve as the initiation site, an initiating methionine can be introduced next to the first codon of the nucleic acid using conventional techniques. Similarly, if the extended cDNA lacks a poly A signal, this sequence can be added to the construct by, for example, splicing out the Poly A signal from pSG5 (Stratagene) using BglI and SalI restriction endonuclease enzymes and incorporating it into the mammalian expression vector pXT1 (Stratagene). pXT1 contains the LTRs and a portion of the *gag* gene from Moloney Murine Leukemia Virus. The position of the LTRs in the construct allow efficient stable transfection. The vector includes the Herpes Simplex Thymidine Kinase promoter and the selectable neomycin gene. The extended cDNA or portion thereof encoding the polypeptide to be expressed is obtained by PCR from the bacterial vector using oligonucleotide primers complementary to the extended cDNA or portion thereof and containing restriction endonuclease sequences for Pst I incorporated into the

5'primer and BglII at the 5' end of the corresponding cDNA 3' primer, taking care to ensure that the extended cDNA is positioned in frame with the poly A signal. The purified fragment obtained from the resulting PCR reaction is digested with PstI, blunt ended with an exonuclease, digested with Bgl II, purified and ligated to pXT1, now containing a poly A signal and digested with BglII.

**[0344]** The ligated product is transfected into mouse NIH 3T3 cells using Lipofectin (Life Technologies, Inc., Grand Island, New York) under conditions outlined in the product specification. Positive transfectants are selected after growing the transfected cells in 600ug/ml G418 (Sigma, St. Louis, Missouri). Preferably the expressed protein is released into the culture medium, thereby facilitating purification.

**[0345]** Alternatively, the extended cDNAs may be cloned into pED6dpc2 as described above. The resulting pED6dpc2 constructs may be transfected into a suitable host cell, such as COS 1 cells. Methotrexate resistant cells are selected and expanded. Preferably, the protein expressed from the extended cDNA is released into the culture medium thereby facilitating purification.

**[0346]** Proteins in the culture medium are separated by gel electrophoresis. If desired, the proteins may be ammonium sulfate precipitated or separated based on size or charge prior to electrophoresis.

**[0347]** As a control, the expression vector lacking a cDNA insert is introduced into host cells or organisms and the proteins in the medium are harvested. The secreted proteins present in the medium are detected using techniques such as Coomassie or silver staining or using antibodies against the protein encoded by the extended cDNA. Coomassie and silver staining techniques are familiar to those skilled in the art.

**[0348]** Antibodies capable of specifically recognizing the protein of interest may be generated using synthetic 15-mer peptides having a sequence encoded by the appropriate 5' EST, extended cDNA, or portion thereof. The synthetic peptides are injected into mice to generate antibody to the polypeptide encoded by the 5' EST, extended cDNA, or portion thereof.

**[0349]** Secreted proteins from the host cells or organisms containing an expression vector which contains the extended cDNA derived from a 5' EST or a portion

thereof are compared to those from the control cells or organism. The presence of a band in the medium from the cells containing the expression vector which is absent in the medium from the control cells indicates that the extended cDNA encodes a secreted protein. Generally, the band corresponding to the protein encoded by the extended cDNA will have a mobility near that expected based on the number of amino acids in the open reading frame of the extended cDNA. However, the band may have a mobility different than that expected as a result of modifications such as glycosylation, ubiquitination, or enzymatic cleavage.

**[0350]** Alternatively, if the protein expressed from the above expression vectors does not contain sequences directing its secretion, the proteins expressed from host cells containing an expression vector containing an insert encoding a secreted protein or portion thereof can be compared to the proteins expressed in host cells containing the expression vector without an insert. The presence of a band in samples from cells containing the expression vector with an insert which is absent in samples from cells containing the expression vector without an insert indicates that the desired protein or portion thereof is being expressed. Generally, the band will have the mobility expected for the secreted protein or portion thereof. However, the band may have a mobility different than that expected as a result of modifications such as glycosylation, ubiquitination, or enzymatic cleavage.

**[0351]** The protein encoded by the extended cDNA may be purified using standard immunochromatography techniques. In such procedures, a solution containing the secreted protein, such as the culture medium or a cell extract, is applied to a column having antibodies against the secreted protein attached to the chromatography matrix. The secreted protein is allowed to bind the immunochromatography column. Thereafter, the column is washed to remove non-specifically bound proteins. The specifically bound secreted protein is then released from the column and recovered using standard techniques.

**[0352]** If antibody production is not possible, the extended cDNA sequence or portion thereof may be incorporated into expression vectors designed for use in purification schemes employing chimeric polypeptides. In such strategies the coding sequence of the extended cDNA or portion thereof is inserted in frame with the gene encoding the other half of the chimera. The other half of the chimera may be β-globin or a

nickel binding polypeptide encoding sequence. A chromatography matrix having antibody to β-globin or nickel attached thereto is then used to purify the chimeric protein. Protease cleavage sites may be engineered between the β-globin gene or the nickel binding polypeptide and the extended cDNA or portion thereof. Thus, the two polypeptides of the chimera may be separated from one another by protease digestion.

**[0353]** One useful expression vector for generating β-globin chimerics is pSG5 (Stratagene), which encodes rabbit β-globin. Intron II of the rabbit β-globin gene facilitates splicing of the expressed transcript, and the polyadenylation signal incorporated into the construct increases the level of expression. These techniques as described are well known to those skilled in the art of molecular biology. Standard methods are published in methods texts such as Davis et al., (**Basic Methods in Molecular Biology**, L.G. Davis, M.D. Dibner, and J.F. Battey, ed., Elsevier Press, NY, 1986) and many of the methods are available from Stratagene, Life Technologies, Inc., or Promega. Polypeptide may additionally be produced from the construct using in vitro translation systems such as the In vitro Express™ Translation Kit (Stratagene).

**[0354]** Following expression and purification of the secreted proteins encoded by the 5′ ESTs, extended cDNAs, or fragments thereof, the purified proteins may be tested for the ability to bind to the surface of various cell types as described in Example 31 below. It will be appreciated that a plurality of proteins expressed from these cDNAs may be included in a panel of proteins to be simultaneously evaluated for the activities specifically described below, as well as other biological roles for which assays for determining activity are available.

## EXAMPLE 31

Analysis of Secreted Proteins to Determine Whether they Bind to the Cell Surface

**[0355]** The proteins encoded by the 5′ ESTs, extended cDNAs, or fragments thereof are cloned into expression vectors such as those described in Example 30. The proteins are purified by size, charge, immunochromatography or other techniques familiar to those skilled in the art. Following purification, the proteins are labeled using techniques known to those skilled in the art. The labeled proteins are incubated with cells

or cell lines derived from a variety of organs or tissues to allow the proteins to bind to any receptor present on the cell surface. Following the incubation, the cells are washed to remove non-specifically bound protein. The labeled proteins are detected by autoradiography. Alternatively, unlabeled proteins may be incubated with the cells and detected with antibodies having a detectable label, such as a fluorescent molecule, attached thereto.

**[0356]** Specificity of cell surface binding may be analyzed by conducting a competition analysis in which various amounts of unlabeled protein are incubated along with the labeled protein. The amount of labeled protein bound to the cell surface decreases as the amount of competitive unlabeled protein increases. As a control, various amounts of an unlabeled protein unrelated to the labeled protein is included in some binding reactions. The amount of labeled protein bound to the cell surface does not decrease in binding reactions containing increasing amounts of unrelated unlabeled protein, indicating that the protein encoded by the cDNA binds specifically to the cell surface.

**[0357]** As discussed above, secreted proteins have been shown to have a number of important physiological effects and, consequently, represent a valuable therapeutic resource. The secreted proteins encoded by the extended cDNAs or portions thereof made according to Examples 27-29 may be evaluated to determine their physiological activities as described below.



## EXAMPLE 32

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Cytokine, Cell Proliferation or Cell Differentiation Activity

**[0358]** As discussed above, secreted proteins may act as cytokines or may affect cellular proliferation or differentiation. Many protein factors discovered to date, including all known cytokines, have exhibited activity in one or more factor dependent cell proliferation assays, and hence the assays serve as a convenient confirmation of cytokine activity. The activity of a protein of the present invention is evidenced by any one of a number of routine factor dependent cell proliferation assays for cell lines including, without limitation, 32D, DA2, DA1G, T10, B9, B9/11, BaF3, MC9/G, M+ (preB M+),

2E8, RB5, DA1, 123, T1165, HT2, CTLL2, TF-1, Mo7c and CMK. The proteins encoded by the above extended cDNAs or portions thereof may be evaluated for their ability to regulate T cell or thymocyte proliferation in assays such as those described above or in the following references, which are incorporated herein by reference: **Current Protocols in Immunology**, Ed. by J.E. Coligan et al., Greene Publishing Associates and Wiley-Interscience; Takai et al. **J. Immunol.** 137:3494-3500, 1986. Bertagnolli et al. **J. Immunol**. 145:1706-1712, 1990. Bertagnolli et al., **Cellular Immunology** 133:327-341, 1991. Bertagnolli, et al. **J. Immunol.** 149:3778-3783, 1992; Bowman et al., **J. Immunol.** 152:1756-1761, 1994.

**[0359]** In addition, numerous assays for cytokine production and/or the proliferation of spleen cells, lymph node cells and thymocytes are known. These include the techniques disclosed in **Current Protocols in Immunology**. J.E. Coligan et al. Eds., Vol 1 pp. 3.12.1-3.12.14 John Wiley and Sons, Toronto. 1994; and Schreiber, R.D. **Current Protocols in Immunology**., *supra* Vol 1 pp. 6.8.1-6.8.8, John Wiley and Sons, Toronto. 1994.

**[0360]** The proteins encoded by the cDNAs may also be assayed for the ability to regulate the proliferation and differentiation of hematopoietic or lymphopoietic cells. Many assays for such activity are familiar to those skilled in the art, including the assays in the following references, which are incorporated herein by reference: Bottomly, K., Davis, L.S. and Lipsky, P.E., Measurement of Human and Murine Interleukin 2 and Interleukin 4, **Current Protocols in Immunology**., J.E. Coligan et al. Eds. Vol 1 pp. 6.3.1-6.3.12, John Wiley and Sons, Toronto. 1991; deVries et al., **J. Exp. Med.** 173:1205-1211, 1991; Moreau et al., **Nature** 36:690-692, 1988; Greenberger et al., **Proc. Natl. Acad. Sci. U.S.A.** 80:2931-2938, 1983; Nordan, R., Measurement of Mouse and Human Interleukin 6 **Current Protocols in Immunology**. J.E. Coligan et al. Eds. Vol 1 pp. 6.6.1-6.6.5, John Wiley and Sons, Toronto. 1991; Smith et al., **Proc. Natl. Acad. Sci. U.S.A.** 83:1857-1861, 1986; Bennett, F., Giannotti, J., Clark, S.C. and Turner, K.J., Measurement of Human Interleukin 11 **Current Protocols in Immunology**. J.E. Coligan et al. Eds. Vol 1 pp. 6.15.1 John Wiley and Sons, Toronto. 1991; Ciarletta, A., Giannotti, J., Clark, S.C. and Turner, K.J., Measurement of Mouse and Human Interleukin 9 **Current Protocols in**

**Immunology**. J.E. Coligan et al., Eds. Vol 1 pp. 6.13.1, John Wiley and Sons, Toronto. 1991.

**[0361]** The proteins encoded by the cDNAs may also be assayed for their ability to regulate T-cell responses to antigens. Many assays for such activity are familiar to those skilled in the art, including the assays described in the following references, which are incorporated herein by reference: Chapter 3 (In Vitro Assays for Mouse Lymphocyte Function), Chapter 6 (Cytokines and Their Cellular Receptors) and Chapter 7, (Immunologic Studies in Humans) in **Current Protocols in Immunology**, J.E. Coligan et al. Eds. Greene Publishing Associates and Wiley-Interscience; Weinberger et al., **Proc. Natl. Acad. Sci. USA** 77:6091-6095, 1980; Weinberger et al., **Eur. J. Immun.** 11:405-411, 1981; Takai et al., **J. Immunol.** 137:3494-3500, 1986; Takai et al., **J. Immunol.** 140:508-512, 1988.

**[0362]** Those proteins which exhibit cytokine, cell proliferation, or cell differentiation activity may then be formulated as pharmaceuticals and used to treat clinical conditions in which induction of cell proliferation or differentiation is beneficial. Alternatively, as described in more detail below, genes encoding these proteins or nucleic acids regulating the expression of these proteins may be introduced into appropriate host cells to increase or decrease the expression of the proteins as desired.

## EXAMPLE 33

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Activity as Immune System Regulators

**[03631]** The proteins encoded by the cDNAs may also be evaluated for their effects as immune regulators. For example, the proteins may be evaluated for their activity to influence thymocyte or splenocyte cytotoxicity. Numerous assays for such activity are familiar to those skilled in the art including the assays described in the following references, which are incorporated herein by reference: Chapter 3 (In Vitro Assays for Mouse Lymphocyte Function 3.1-3.19) and Chapter 7 (Immunologic studies in Humans) in **Current Protocols in Immunology**, J.E. Coligan et al. Eds, Greene Publishing Associates and Wiley-Interscience; Herrmann et al., **Proc. Natl. Acad. Sci. USA** 78:2488-

2492, 1981; Herrmann et al., **J. Immunol.** 128:1968-1974, 1982; Handa et al., **J. Immunol.** 135:1564-1572, 1985; Takai et al., **J. Immunol.** 137:3494-3500, 1986; Takai et al., **J. Immunol.** 140:508-512, 1988; Herrmann et al., **Proc. Natl. Acad. Sci. USA** 78:2488-2492, 1981; Herrmann et al., **J. Immunol.** 128:1968-1974, 1982; Handa et al., **J. Immunol.** 135:1564-1572, 1985; Takai et al., **J. Immunol.** 137:3494-3500, 1986; Bowman et al., **J. Virology** 61:1992-1998; Takai et al., **J. Immunol.** 140:508-512, 1988; Bertagnolli et al., **Cellular Immunology** 133:327-341, 1991; Brown et al., **J. Immunol.** 153:3079-3092, 1994.

**[0364]** The proteins encoded by the cDNAs may also be evaluated for their effects on T-cell dependent immunoglobulin responses and isotype switching. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Maliszewski, **J. Immunol.** 144:3028-3033, 1990; Mond, J.J. and Brunswick, M Assays for B Cell Function: *In vitro* Antibody Production, Vol 1 pp. 3.8.1-3.8.16 in **Current Protocols in Immunology.** J.E. Coligan et al Eds., John Wiley and Sons, Toronto. 1994.

**[0365]** The proteins encoded by the cDNAs may also be evaluated for their effect on immune effector cells, including their effect on Th1 cells and cytotoxic lymphocytes. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Chapter 3 (In Vitro Assays for Mouse Lymphocyte Function 3.1-3.19) and Chapter 7 (Immunologic Studies in Humans) in **Current Protocols in Immunology**, J.E. Coligan et al. Eds., Greene Publishing Associates and Wiley-Interscience; Takai et al., **J. Immunol.** 137:3494-3500, 1986; Takai et al.; **J. Immunol.** 140:508-512, 1988; Bertagnolli et al., **J. Immunol.** 149:3778-3783, 1992.

**[0366]** The proteins encoded by the cDNAs may also be evaluated for their effect on dendritic cell mediated activation of naive T-cells. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Guery et al., **J. Immunol.** 134:536-544, 1995; Inaba et al., **Journal of Experimental Medicine** 173:549-559, 1991; Macatonia et al., **Journal of Immunology** 154:5071-5079, 1995; Porgador et al., **Journal of Experimental Medicine** 182:255-260, 1995; Nair et al., **Journal of**

**Virology** 67:4062-4069, 1993; Huang et al., **Science** 264:961-965, 1994; Macatonia et al., **Journal of Experimental Medicine** 169:1255-1264, 1989; Bhardwaj et al., **Journal of Clinical Investigation** 94:797-807, 1994; and Inaba et al., **Journal of Experimental Medicine** 172:631-640, 1990.

**[0367]** The proteins encoded by the cDNAs may also be evaluated for their influence on the lifetime of lymphocytes. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Darzynkiewicz et al., **Cytometry** 13:795-808, 1992; Gorczyca et al., **Leukemia** 7:659-670, 1993; Gorczyca et al., **Cancer Research** 53:1945-1951, 1993; Itoh et al., **Cell** 66:233-243, 1991; Zacharchuk, **Journal of Immunology** 145:4037-4045, 1990; Zamai et al., Cytometry 14:891-897, 1993; Gorczyca et al., **International Journal of Oncology** 1:639-648, 1992.

**[0368]** Assays for proteins that influence early steps of T-cell commitment and development include, without limitation, those described in: Antica et al., Blood 84:111-117, 1994; Fine et al., Cellular immunology 155:111-122, 1994; Galy et al., Blood 85:2770-2778, 1995; Toki et al., Proc. Nat. Acad Sci. USA 88:7548-7551, 1991.

**[0369]** Those proteins which exhibit activity as immune system regulators activity may then be formulated as pharmaceuticals and used to treat clinical conditions in which regulation of immune activity is beneficial. For example, the protein may be useful in the treatment of various immune deficiencies and disorders (including severe combined immunodeficiency (SCID)), e.g., in regulating (up or down) growth and proliferation of T and/or B lymphocytes, as well as effecting the cytolytic activity of NK cells and other cell populations. These immune deficiencies may be genetic or be caused by viral (e.g., HIV) as well as bacterial or fungal infections, or may result from autoimmune disorders. More specifically, infectious diseases caused by viral, bacterial, fungal or other infection may be treatable using a protein of the present invention, including infections by HIV, hepatitis viruses, herpesviruses, mycobacteria, Leishmania spp., malaria spp. and various fungal infections such as candidiasis. Of course, in this regard, a protein of the present invention may also be useful where a boost to the immune system generally may be desirable, i.e., in the treatment of cancer.

**[0370]** Autoimmune disorders which may be treated using a protein of the present invention include, for example, connective tissue disease, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome, autoimmune thyroiditis, insulin dependent diabetes mellitis, myasthenia gravis, graft-versus-host disease and autoimmune inflammatory eye disease. Such a protein of the present invention may also to be useful in the treatment of allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems. Other conditions, in which immune suppression is desired (including, for example, organ transplantation), may also be treatable using a protein of the present invention.

**[0371]** Using the proteins of the invention it may also be possible to regulate immune responses, in a number of ways. Down regulation may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The functions of activated T-cells may be inhibited by suppressing T cell responses or by inducing specific tolerance in T cells, or both. Immunosuppression of T cell responses is generally an active, non-antigen-specific, process which requires continuous exposure of the T cells to the suppressive agent. Tolerance, which involves inducing non-responsiveness or anergy in T cells, is distinguishable from immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, tolerance can be demonstrated by the lack of a T cell response upon reexposure to specific antigen in the absence of the tolerizing agent.

**[0372]** Down regulating or preventing one or more antigen functions (including without limitation B lymphocyte antigen functions (such as, for example, B7)), e.g., preventing high level lymphokine synthesis by activated T cells, will be useful in situations of tissue, skin and organ transplantation and in graft-versus-host disease (GVHD). For example, blockage of T cell function should result in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by T cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits or blocks interaction of a B7 lymphocyte antigen with its natural ligand(s) on immune cells

(such as a soluble, monomeric form of a peptide having B7-2 activity alone or in conjunction with a monomeric form of a peptide having an activity of another B lymphocyte antigen (e.g., B7-1, B7-3) or blocking antibody), prior to transplantation can lead to the binding of the molecule to the natural ligand(s) on the immune cells without transmitting the corresponding costimulatory signal. Blocking B lymphocyte antigen function in this matter prevents cytokine synthesis by immune cells, such as T cells, and thus acts as an immunosuppressant. Moreover, the lack of costimulation may also be sufficient to anergize the T cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by B lymphocyte antigen-blocking reagents may avoid the necessity of repeated administration of these blocking reagents. To achieve sufficient immunosuppression or tolerance in a subject, it may also be necessary to block the function of a combination of B lymphocyte antigens.

**[0373]** The efficacy of particular blocking reagents in preventing organ transplant rejection or GVHD can be assessed using animal models that are predictive of efficacy in humans. Examples of appropriate systems which can be used include allogeneic cardiac grafts in rats and xenogeneic pancreatic islet cell grafts in mice, both of which have been used to examine the immunosuppressive effects of CTLA4Ig fusion proteins in vivo as described in Lenschow et al., Science 257:789-792 (1992) and Turka et al., Proc. Natl. Acad. Sci USA, 89:11102-11105 (1992). In addition, murine models of GVHD (see Paul ed., Fundamental Immunology, Raven Press, New York, 1989, pp. 846-847) can be used to determine the effect of blocking B lymphocyte antigen function in vivo on the development of that disease.

**[0374]** Blocking antigen function may also be therapeutically useful for treating autoimmune diseases. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive T cells may reduce or eliminate disease symptoms. Administration of reagents which block costimulation of T cells by disrupting receptor ligand interactions of B lymphocyte antigens can be used to inhibit T cell activation and prevent production of autoantibodies or T cell-derived cytokines which may be involved in the disease process. Additionally, blocking reagents may induce antigen-specific tolerance

of autoreactive T cells which could lead to long-term relief from the disease. The efficacy of blocking reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythmatosis in MRL/pr/pr mice or NZB hybrid mice, murine autoimmuno collagen arthritis, diabetes mellitus in OD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., Fundamental Immunology, Raven Press, New York, 1989, pp. 840-856).

**[0375]** Upregulation of an antigen function (preferably a B lymphocyte antigen function), as a means of up regulating immune responses, may also be useful in therapy. Upregulation of immune responses may be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response through stimulating B lymphocyte antigen function may be useful in cases of viral infection. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the administration of stimulatory form of B lymphocyte antigens systemically.

**[0376]** Alternatively, anti-viral immune responses may be enhanced in an infected patient by removing T cells from the patient, costimulating the T cells in vitro with viral antigen-pulsed APCs either expressing a peptide of the present invention or together with a stimulatory form of a soluble peptide of the present invention and reintroducing the in vitro activated T cells into the patient. The infected cells would now be capable of delivering a costimulatory signal to T cells in vivo, thereby activating the T cells.

**[0377]** In another application, up regulation or enhancement of antigen function (preferably B lymphocyte antigen function) may be useful in the induction of tumor immunity. Tumor cells (e.g., sarcoma, melanoma, lymphoma, leukemia, neuroblastoma, carcinoma) transfected with a nucleic acid encoding at least one peptide of the present invention can be administered to a subject to overcome tumor-specific tolerance in the subject. If desired, the tumor cell can be transfected to express a combination of peptides. For example, tumor cells obtained from a patient can be transfected ex vivo with an expression vector directing the expression of a peptide having

B7-2-like activity alone, or in conjunction with a peptide having B7-1-like activity and/or B7-3-like activity. The transfected tumor cells are returned to the patient to result in expression of the peptides on the surface of the transfected cell. Alternatively, gene therapy techniques can be used to target a tumor cell for transfection in vivo.

**[0378]** The presence of the peptide of the present invention having the activity of a B lymphocyte antigen(s) on the surface of the tumor cell provides the necessary costimulation signal to T cells to induce a T cell mediated immune response against the transfected tumor cells. In addition, tumor cells which lack MHC class I or MHC class II molecules, or which fail to reexpress sufficient amounts of MHC class I or MHC class II molecules, can be transfected with nucleic acids encoding all or a portion of (e.g., a cytoplasmic-domain truncated portion) of an MHC class I $\alpha$ chain protein and $\beta_2$ macroglobulin protein or an MHC class II $\alpha$ chain protein and an MHC class II $\beta$ chain protein to thereby express MHC class I or MHC class II proteins on the cell surface. Expression of the appropriate class II or class II MHC in conjunction with a peptide having the activity of a B lymphocyte antigen (e.g., B7-1, B7-2, B7-3) induces a T cell mediated immune response against the transfected tumor cell. Optionally, a gene encoding an antisense construct which blocks expression of an MHC class II associated protein, such as the invariant chain, can also be cotransfected with a DNA encoding a peptide having the activity of a B lymphocyte antigen to promote presentation of tumor associated antigens and induce tumor specific immunity. Thus, the induction of a T cell mediated immune response in a human subject may be sufficient to overcome tumor-specific tolerance in the subject. Alternatively, as described in more detail below, genes encoding these proteins or nucleic acids regulating the expression of these proteins may be introduced into appropriate host cells to increase or decrease the expression of the proteins as desired.



## EXAMPLE 34

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Hematopoiesis Regulating Activity

**[0379]** The proteins encoded by the extended cDNAs or portions thereof may also be evaluated for their hematopoiesis regulating activity. For example, the effect

of the proteins on embryonic stem cell differentiation may be evaluated. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Johansson et al. **Cellular Biology** 15:141-151, 1995; Keller et al., **Molecular and Cellular Biology** 13:473-486, 1993; McClanahan et al., **Blood** 81:2903-2915, 1993.

**[0380]** The proteins encoded by the extended cDNAs or portions thereof may also be evaluated for their influence on the lifetime of stem cells and stem cell differentiation. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Freshney, M.G. Methylcellulose Colony Forming Assays, in **Culture of Hematopoietic Cells**. R.I. Freshney, et al. Eds. pp. 265-268, Wiley-Liss, Inc., New York, NY. 1994; Hirayama et al., **Proc. Natl. Acad. Sci. USA** 89:5907-5911, 1992; McNiece, I.K. and Briddell, R.A. Primitive Hematopoietic Colony Forming Cells with High Proliferative Potential, in **Culture of Hematopoietic Cells**. R.I. Freshney, et al. eds. Vol pp. 23-39, Wiley-Liss, Inc., New York, NY. 1994; Neben et al., **Experimental Hematology** 22:353-359, 1994; Ploemacher, R.E. Cobblestone Area Forming Cell Assay, In **Culture of Hematopoietic Cells.** R.I. Freshney, et al. Eds. pp. 1-21, Wiley-Liss, Inc., New York, NY. 1994; Spooncer, E., Dexter, M. and Allen, T. Long Term Bone Marrow Cultures in the Presence of Stromal Cells, in **Culture of Hematopoietic Cells**. R.I. Freshney, et al. Eds. pp. 163-179, Wiley-Liss, Inc., New York, NY. 1994; and Sutherland, H.J. Long Term Culture Initiating Cell Assay, in **Culture of Hematopoietic Cells**. R.I. Freshney, et al. Eds. pp. 139-162, Wiley-Liss, Inc., New York, NY. 1994.

**[0381]** Those proteins which exhibit hematopoiesis regulatory activity may then be formulated as pharmaceuticals and used to treat clinical conditions in which regulation of hematopoeisis is beneficial. For example, a protein of the present invention may be useful in regulation of hematopoiesis and, consequently, in the treatment of myeloid or lymphoid cell deficiencies. Even marginal biological activity in support of colony forming cells or of factor-dependent cell lines indicates involvement in regulating hematopoiesis, e.g. in supporting the growth and proliferation of erythroid progenitor cells alone or in combination with other cytokines, thereby indicating utility, for example, in treating various anemias or for use in conjunction with irradiation/chemotherapy to

stimulate the production of erythroid precursors and/or erythroid cells; in supporting the growth and proliferation of myeloid cells such as granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelo-suppression; in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentary to platelet transfusions; and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility in various stem cell disorders (such as those usually treated with transplantion, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either in-vivo or ex-vivo (i.e., in conjunction with bone marrow transplantation or with peripheral progenitor cell transplantation (homologous or heterologous)) as normal cells or genetically manipulated for gene therapy. Alternatively, as described in more detail below, genes encoding these proteins or nucleic acids regulating the expression of these proteins may be introduced into appropriate host cells to increase or decrease the expression of the proteins as desired.



## EXAMPLE 35

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Regulation of Tissue Growth

**[0382]** The proteins encoded by the extended cDNAs or portions thereof may also be evaluated for their effect on tissue growth. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in International Patent Publication No. WO95/16035, International Patent Publication No. WO95/05846 and International Patent Publication No. WO91/07491, which are incorporated herein by reference.

**[0383]** Assays for wound healing activity include, without limitation, those described in: Winter, Epidermal Wound Healing, pps. 71-112 (Maibach, H1 and Rovee,

DT, eds.), Year Book Medical Publishers, Inc., Chicago, as modified by Eaglstein and Mertz, J. Invest. Dermatol 71:382-84 (1978) which are incorporated herein by reference.

**[0384]** Those proteins which are involved in the regulation of tissue growth may then be formulated as pharmaceuticals and used to treat clinical conditions in which regulation of tissue growth is beneficial. For example, a protein of the present invention also may have utility in compositions used for bone, cartilage, tendon, ligament and/or nerve tissue growth or regeneration, as well as for wound healing and tissue repair and replacement, and in the treatment of burns, incisions and ulcers.

**[0385]** A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage damage or defects in humans and other animals. Such a preparation employing a protein of the invention may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery.

**[0386]** A protein of this invention may also be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A protein of the invention may also be useful in the treatment of osteoporosis or osteoarthritis, such as through stimulation of bone and/or cartilage repair or by blocking inflammation or processes of tissue destruction (collagenase activity, osteoclast activity, etc.) mediated by inflammatory processes.

**[0387]** Another category of tissue regeneration activity that may be attributable to the protein of the present invention is tendon/ligament formation. A protein of the present invention, which induces tendon/ligament-like tissue or other tissue formation in circumstances where such tissue is not normally formed, has application in the healing of tendon or ligament tears, deformities and other tendon or ligament defects in humans and other animals. Such a preparation employing a tendon/ligament-like tissue inducing protein may have prophylactic use in preventing damage to tendon or ligament

tissue, as well as use in the improved fixation of tendon or ligament to bone or other tissues, and in repairing defects to tendon or ligament tissue. De novo tendon/ligament-like tissue formation induced by a composition of the present invention contributes to the repair of congenital, trauma induced, or other tendon or ligament defects of other origin, and is also useful in cosmetic plastic surgery for attachment or repair of tendons or ligaments. The compositions of the present invention may provide an environment to attract tendon- or ligament-forming cells, stimulate growth of tendon- or ligament-forming cells, induce differentiation of progenitors of tendon- or ligament-forming cells, or induce growth of tendon/ligament cells or progenitors ex vivo for return in vivo to effect tissue repair. The compositions of the invention may also be useful in the treatment of tendinitis, carpal tunnel syndrome and other tendon or ligament defects. The compositions may also include an appropriate matrix and/or sequestering agent as a carrier as is well known in the art.

**[0388]** The protein of the present invention may also be useful for proliferation of neural cells and for regeneration of nerve and brain tissue, i.e., for the treatment of central and peripheral nervous system diseases and neuropathies, as well as mechanical and traumatic disorders, which involve degeneration, death or trauma to neural cells or nerve tissue. More specifically, a protein may be used in the treatment of diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome. Further conditions which may be treated in accordance with the present invention include mechanical and traumatic disorders, such as spinal cord disorders, head trauma and cerebrovascular diseases such as stroke. Peripheral neuropathies resulting from chemotherapy or other medical therapies may also be treatable using a protein of the invention.

**[0389]** Proteins of the invention may also be useful to promote better or faster closure of non-healing wounds, including without limitation pressure ulcers, ulcers associated with vascular insufficiency, surgical and traumatic wounds, and the like.

**[0390]** It is expected that a protein of the present invention may also exhibit activity for generation or regeneration of other tissues, such as organs (including,

for example, pancreas, liver, intestine, kidney, skin, endothelium) muscle (smooth, skeletal or cardiac) and vascular (including vascular endothelium) tissue, or for promoting the growth of cells comprising such tissues. Part of the desired effects may be by inhibition or modulation of fibrotic scarring to allow normal tissue to generate. A protein of the invention may also exhibit angiogenic activity.

**[0391]** A protein of the present invention may also be useful for gut protection or regeneration and treatment of lung or liver fibrosis, reperfusion injury in various tissues, and conditions resulting from systemic cytokinc damage.

**[0392]** A protein of the present invention may also be useful for promoting or inhibiting differentiation of tissues described above from precursor tissues or cells; or for inhibiting the growth of tissues described above.

**[0393]** Alternatively, as described in more detail below, genes encoding these proteins or nucleic acids regulating the expression of these proteins may be introduced into appropriate host cells to increase or decrease the expression of the proteins as desired.



## EXAMPLE 36

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Regulation of Reproductive Hormones or Cell Movement

**[0394]** The proteins encoded by the extended cDNAs or portions thereof may also be evaluated for their ability to regulate reproductive hormones, such as follicle stimulating hormone. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Vale et al., **Endocrinology** 91:562-572, 1972; Ling et al., **Nature** 321:779-782, 1986; Vale et al., **Nature** 321:776-779, 1986; Mason et al., **Nature** 318:659-663, 1985; Forage et al., **Proc. Natl. Acad. Sci. USA** 83:3091-3095, 1986. Chapter 6.12 (Measurement of Alpha and Beta Chemokines) **Current Protocols in Immunology**, J.E. Coligan et al. Eds. Greene Publishing Associates and Wiley-Intersciece ; Taub et al. **J. Clin. Invest.** 95:1370-1376, 1995; Lind et al. **APMIS** 103:140-146, 1995; Muller et al.

**Eur. J. Immunol.** 25:1744-1748; Gruber et al. **J. of Immunol.** 152:5860-5867, 1994; Johnston et al. **J. of Immunol.** 153:1762-1768, 1994.

**[0395]** Those proteins which exhibit activity as reproductive hormones or regulators of cell movement may then be formulated as pharmaceuticals and used to treat clinical conditions in which regulation of reproductive hormones or cell movement are beneficial. For example, a protein of the present invention may also exhibit activin- or inhibin-related activities. Inhibins are characterized by their ability to inhibit the release of follicle stimulating hormone (FSH), while activins are characterized by their ability to stimulate the release of folic stimulating hormone (FSH). Thus, a protein of the present invention, alone or in heterodimers with a member of the inhibin α family, may be useful as a contraceptive based on the ability of inhibins to decrease fertility in female mammals and decrease spermatogenesis in male mammals. Administration of sufficient amounts of other inhibins can induce infertility in these mammals. Alternatively, the protein of the invention, as a homodimer or as a heterodimer with other protein subunits of the inhibin-B group, may be useful as a fertility inducing therapeutic, based upon the ability of activin molecules in stimulating FSH release from cells of the anterior pituitary. See, for example, United States Patent 4,798,885, the disclosure of which is incorporated herein by reference. A protein of the invention may also be useful for advancement of the onset of fertility in sexually immature mammals, so as to increase the lifetime reproductive performance of domestic animals such as cows, sheep and pigs.

**[0396]** Alternatively, as described in more detail below, genes encoding these proteins or nucleic acids regulating the expression of these proteins may be introduced into appropriate host cells to increase or decrease the expression of the proteins as desired.

## EXAMPLE 36A

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Chemotactic/Chemokinetic Activity

**[0397]** The proteins encoded by the extended cDNAs or portions thereof may also be evaluated for chemotactic/chemokinetic activity. For example, a protein of

the present invention may have chemotactic or chemokinetic activity (e.g., act as a chemokine) for mammalian cells, including, for example, monocytes, fibroblasts, neutrophils, T-cells, mast cells, cosinophils, epithelial and/or endothelial cells. Chemotactic and chmokinetic proteins can be used to mobilize or attract a desired cell population to a desired site of action. Chemotactic or chemokinetic proteins provide particular advantages in treatment of wounds and other trauma to tissues, as well as in treatment of localized infections. For example, attraction of lymphocytes, monocytes or neutrophils to tumors or sites of infection may result in improved immune responses against the tumor or infecting agent.

**[0398]** A protein or peptide has chemotactic activity for a particular cell population if it can stimulate, directly or indirectly, the directed orientation or movement of such cell population. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells. Whether a particular protein has chemotactic activity for a population of cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

**[0399]** The activity of a protein of the invention may, among other means, be measured by the following methods:

**[0400]** Assays for chemotactic activity (which will identify proteins that induce or prevent chemotaxis) consist of assays that measure the ability of a protein to induce the migration of cells across a membrane as well as the ability of a protein to induce the adhension of one cell population to another cell population. Suitable assays for movement and adhesion include, without limitation, those described in: Current Protocols in Immunology, Ed by J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 6.12, Measurement of alpha and beta Chemokincs 6.12.1-6.12.28; Taub et al. J. Clin. Invest. 95:1370-1376, 1995; Lind et al. APMIS 103:140-146, 1995; Mueller et al Eur. J. Immunol. 25:1744-1748; Gruber et al. J. of Immunol. 152:5860-5867, 1994; Johnston et al. J. of Immunol, 153:1762-1768, 1994.

## EXAMPLE 37

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Regulation of Blood Clotting

**[0401]** The proteins encoded by the extended cDNAs or portions thereof may also be evaluated for their effects on blood clotting. Numerous assays for such activity are familiar to those skilled in the art, including the assays disclosed in the following references, which are incorporated herein by reference: Linet et al., **J. Clin. Pharmacol.** 26:131-140, 1986; Burdick et al., **Thrombosis Res.** 45:413-419, 1987; Humphrey et al., **Fibrinolysis** 5:71-79 (1991); Schaub, **Prostaglandins** 35:467-474, 1988.

**[0402]** Those proteins which are involved in the regulation of blood clotting may then be formulated as pharmaceuticals and used to treat clinical conditions in which regulation of blood clotting is beneficial. For example, a protein of the invention may also exhibit hemostatic or thrombolytic activity. As a result, such a protein is expected to be useful in treatment of various coagulations disorders (including hereditary disorders, such as hemophilias) or to enhance coagulation and other hemostatic events in treating wounds resulting from trauma, surgery or other causes. A protein of the invention may also be useful for dissolving or inhibiting formation of thromboses and for treatment and prevention of conditions resulting therefrom (such as, for example, infarction of cardiac and central nervous system vessels (e.g., stroke). Alternatively, as described in more detail below, genes encoding these proteins or nucleic acids regulating the expression of these proteins may be introduced into appropriate host cells to increase or decrease the expression of the proteins as desired.

## EXAMPLE 38

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Involvement in Receptor/Ligand Interactions

**[0403]** The proteins encoded by the extended cDNAs or a portion thereof may also be evaluated for their involvement in receptor/ligand interactions. Numerous assays for such involvement are familiar to those skilled in the art, including the assays

disclosed in the following references, which are incorporated herein by reference: Chapter 7.28 (Measurement of Cellular Adhesion under Static Conditions 7.28.1-7.28.22) in **Current Protocols in Immunology**, J.E. Coligan et al. Eds. Greene Publishing Associates and Wiley-Interscience; Takai et al., **Proc. Natl. Acad. Sci. USA** 84:6864-6868, 1987; Bierer et al., **J. Exp. Med.** 168:1145-1156, 1988; Rosenstein et al., **J. Exp. Med.** 169:149-160, 1989; Stoltenborg et al., **J. Immunol. Methods** 175:59-68, 1994; Stitt et al., **Cell** 80:661-670, 1995; Gyuris et al., *Cell* 75:791-803, 1993.

**[0404]** For example, the proteins of the present invention may also demonstrate activity as receptors, receptor ligands or inhibitors or agonists of receptor/ligand interactions. Examples of such receptors and ligands include, without limitation, cytokine receptors and their ligands, receptor kinases and their ligands, receptor phosphatases and their ligands, receptors involved in cell-cell interactions and their ligands (including without limitation, cellular adhesion molecules (such as selectins, integrins and their ligands) and receptor/ligand pairs involved in antigen presentation, antigen recognition and development of cellular and humoral immune respones). Receptors and ligands are also useful for screening of potential peptide or small molecule inhibitors of the relevant receptor/ligand interaction. A protein of the present invention (including, without limitation, fragments of receptors and ligands) may themselves be useful as inhibitors of receptor/ligand interactions.



## EXAMPLE 38A

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Anti-Inflammatory Activity

**[0405]** The proteins encoded by the extended cDNAs or a portion thereof may also be evaluated for anti-inflammatory activity. The anti-inflammatory activity may be achieved by providing a stimulus to cells involved in the inflammatory response, by inhibiting or promoting cell-cell interactions (such as, for example, cell adhesion), by inhibiting or promoting chemotaxis of cells involved in the inflammatory process, inhibiting or promoting cell extravasation, or by stimulating or suppressing production of other factors which more directly inhibit or promote an inflammatory response. Proteins

exhibiting such activities can be used to treat inflammatory conditions including chronic or acute conditions), including without limitation inflammation associated with infection (such as septic shock, sepsis or systemic inflammatory response syndrome (SIRS)),ischemia-reperfusioninury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine-induced lung injury, inflammatory bowel disease, Crohn's disease or resulting from over production of cytokines such as TNF or IL-1. Proteins of the invention may also be useful to treat anaphylaxis and hypersensitivity to an antigenic substance or material.

## EXAMPLE 38B

Assaying the Proteins Expressed from Extended cDNAs or Portions Thereof for Tumor Inhibition Activity

**[0406]** The proteins encoded by the extended cDNAs or a portion thereof may also be evaluated for tumor inhibition activity. In addition to the activities described above for immunological treatment or prevention of tumors, a protein of the invention may exhibit other anti-tumor activities. A protein may inhibit tumor growth directly or indirectly (such as, for example, via ADCC). A protein may exhibit its tumor inhibitory activity by acting on tumor tissue or tumor precursor tissue, by inhibiting formation of tissues necessary to support tumor growth (such as, for example, by inhibiting angiogenesis), by causing production of other factors, agents or cell types which inhibit tumor growth, or by suppressing, climinating or inhibiting factors, agents or cell types which promote tumor growth.

**[0407]** A protein of the invention may also exhibit one or more of the following additional activities or effects: inhibiting the growth, infection or function of, or killing, infectious agents, including, without limitation, bacteria, viruses, fungi and other parasites; effecting (suppressing or enhancing) bodily characteristics, including, without limitation, height, weight, hair color, eye color, skin, fat to lean ratio or other tissue pigmentation, or organ or body part size or shape (such as, for example, breast augmentation or diminution, change in bone form or shape); effecting biorhythms or circadian cycles or rhythms; effecting the fertility of male or female subjects; effecting the

metabolism, catabolism, anabolism, processing, utilization, storage or climination of dietary fat, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional factors or component(s); effecting behavioral characteristics, including, without limitation, appetite, libido, stress, cognition (including cognitive disorders), depression (including depressive disorders) and violent behaviors; providing analgesic effects or other pain reducing effects; promoting differentiation and growth of embryonic stem cells in lineages other than hematopoietic lineages; hormonal or endocrine activity; in the case of enzymes, correcting deficiencies of the enzyme and treating deficiency-related diseases; treatment of hyperproliferative disorders (such as, for example, psoriasis); immunoglobulin-like activity (such as, for example, the ability to bind antigens or complement); and the ability to act as an antigen in a vaccine composition to raise an immune response against such protein or another material or entity which is cross-reactive with such protein.



**EXAMPLE 39**

Identification of Proteins which Interact with

Polypeptides Encoded by Extended cDNAs

**[0408]** Proteins which interact with the polypeptides encoded by extended cDNAs or portions thereof, such as receptor proteins, may be identified using two hybrid systems such as the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech). As described in the manual accompanying the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech), which is incorporated herein by reference, the extended cDNAs or portions thereof, are inserted into an expression vector such that they are in frame with DNA encoding the DNA binding domain of the yeast transcriptional activator GAL4. cDNAs in a cDNA library which encode proteins which might interact with the polypeptides encoded by the extended cDNAs or portions thereof are inserted into a second expression vector such that they are in frame with DNA encoding the activation domain of GAL4. The two expression plasmids are transformed into yeast and the yeast are plated on selection medium which selects for expression of selectable markers on each of the expression vectors as well as GAL4 dependent expression of the HIS3 gene. Transformants capable of growing on medium lacking histidine are screened for GAL4

dependent lacZ expression. Those cells which are positive in both the histidine selection and the lacZ assay contain plasmids encoding proteins which interact with the polypeptide encoded by the extended cDNAs or portions thereof.

**[0409]** Alternatively, the system described in Lustig et al., Methods in Enzymology 283: 83-99 (1997), the disclosure of which is incorporated herein by reference, may be used for identifying molecules which interact with the polypeptides encoded by extended cDNAs. In such systems, *in vitro* transcription reactions are performed on a pool of vectors containing extended cDNA inserts cloned downstream of a promoter which drives *in vitro* transcription. The resulting pools of mRNAs are introduced into *Xenopus laevis* oocytes. The oocytes are then assayed for a desired acitivity.

**[0410]** Alternatively, the pooled *in vitro* transcription products produced as described above may be translated *in vitro*. The pooled *in vitro* translation products can be assayed for a desired activity or for interaction with a known polypeptide.

**[0411]** Proteins or other molecules interacting with polypeptides encoded by extended cDNAs can be found by a variety of additional techniques. In one method, affinity columns containing the polypeptide encoded by the extended cDNA or a portion thereof can be constructed. In some versions, of this method the affinity column contains chimeric proteins in which the protein encoded by the extended cDNA or a portion thereof is fused to glutathione S-transferase. A mixture of cellular proteins or pool of expressed proteins as described above and is applied to the affinity column. Proteins interacting with the polypeptide attached to the column can then be isolated and analyzed on 2-D electrophoresis gel as described in Ramunsen et al. Electrophoresis, 18, 588-598 (1997), the disclosure of which is incorporated herein by reference. Alternatively, the proteins retained on the affinity column can be purified by electrophoresis based methods and sequenced. The same method can be used to isolate antibodies, to screen phage display products, or to screen phage display human antibodies.

**[0412]** Proteins interacting with polypeptides encoded by extended cDNAs or portions thereof can also be screened by using an Optical Biosensor as described in Edwards & Leatherbarrow, Analytical Biochemistry, 246, 1-6 (1997), the

disclosure of which is incorporated herein by reference. The main advantage of the method is that it allows the determination of the association rate between the protein and other interacting molecules. Thus, it is possible to specifically select interacting molecules with a high or low association rate. Typically a target molecule is linked to the sensor surface (through a carboxymethl dextran matrix) and a sample of test molecules is placed in contact with the target molecules. The binding of a test molecule to the target molecule causes a change in the refractive index and/ or thickness. This change is detected by the Biosensor provided it occurs in the evanescent field (which extend a few hundred manometers from the sensor surface). In these screening assays, the target molecule can be one of the polypeptides encoded by extended cDNAs or a portion thereof and the test sample can be a collection of proteins extracted from tissues or cells, a pool of expressed proteins, combinatorial peptide and/ or chemical libraries,or phage displayed peptides. The tissues or cells from which the test proteins are extracted can originate from any species.

**[0413]** In other methods, a target protein is immobilized and the test population is a collection of unique polypeptides encoded by the extended cDNAs or portions thereof.

**[0414]** To study the interaction of the proteins encoded by the extended cDNAs or portions thereof with drugs, the microdialysis coupled to HPLC method described by Wang et al., Chromatographia, 44, 205-208(1997) or the affinity capillary electrophoresis method described by Busch et al., J. Chromatogr. 777:311-328 (1997), the disclosures of which are incorporated herein by referenc can be used.

**[0415]** The system described in U.S. Patent No. 5,654,150, the disclosure of which is incorporated herein by reference, may also be used to identify molecules which interact with the polypeptides encoded by the extended cDNAs. In this system, pools of extended cDNAs are transcribed and translated *in vitro* and the reaction products are assayed for interaction with a known polypeptide or antibody.

**[0416]** It will be appreciated by those skilled in the art that the proteins expressed from the extended cDNAs or portions may be assayed for numerous activities in addition to those specifically enumerated above. For example, the expressed proteins may be evaluated for applications involving control and regulation of inflammation, tumor

proliferation or metastasis, infection, or other clinical conditions. In addition, the proteins expressed from the extended cDNAs or portions thereof may be useful as nutritional agents or cosmetic agents.

**[0417]** The proteins expressed from the extended cDNAs or portions thereof may be used to generate antibodies capable of specifically binding to the expressed protein or fragments thereof as described in Example 40 below. The antibodies may be capable of binding a full length protein encoded by one of the sequences of SEQ ID NOs: 40-59, 61-73, 75, 77-82, and 130-154, a mature protein encoded by one of the sequences of SEQ ID NOs. 40-59, 61-75, 77-82, and 130-154, or a signal peptide encoded by one of the sequences of SEQ ID Nos. 40-59, 61-73, 75-82, 84 and 130-154. Alternatively, the antibodies may be capable of binding fragments of the proteins expressed from the extended cDNAs which comprise at least 10 amino acids of the sequences of SEQ ID NOs: 85-129 and 155-179. In some embodiments, the antibodies may be capable of binding fragments of the proteins expressed from the extended cDNAs which comprise at least 15 amino acids of the sequences of SEQ ID NOs: 85-129 and 155-179. In other embodiments, the antibodies may be capable of binding fragments of the proteins expressed from the extended cDNAs which comprise at least 25 amino acids of the sequences of SEQ ID NOs: 85-129 and 155-179. In further embodiments, the antibodies may be capable of binding fragments of the proteins expressed from the extended cDNAs which comprise at least 40 amino acids of the sequences of SEQ ID NOs: 85-129 and 155-179.

## EXAMPLE 40

Production of an Antibody to a Human Protein

**[0418]** Substantially pure protein or polypeptide is isolated from the transfected or transformed cells as described in Example 30. The concentration of protein in the final preparation is adjusted, for example, by concentration on an Amicon filter device, to the level of a few micrograms/ml. Monoclonal or polyclonal antibody to the protein can then be prepared as follows:

### A. Monoclonal Antibody Production by Hybridoma Fusion

**[0419]** Monoclonal antibody to epitopes of any of the peptides identified and isolated as described can be prepared from murine hybridomas according to the classical method of Kohler, G. and Milstein, C., **Nature 256**:495 (1975) or derivative methods thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the selected protein or peptides derived therefrom over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as Elisa, as originally described by Engvall, E., **Meth. Enzymol. 70**:419 (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. et al. **Basic Methods in Molecular Biology** Elsevier, New York. Section 21-2.

### B. Polyclonal Antibody Production by Immunization

**[0420]** Polyclonal antiserum containing antibodies to heterogeneous epitopes of a single protein can be prepared by immunizing suitable animals with the expressed protein or peptides derived therefrom described above, which can be unmodified or modified to enhance immunogenicity. Effective polyclonal antibody production is

affected by many factors related both to the antigen and the host species. For example, small molecules tend to be less immunogenic than others and may require the use of carriers and adjuvant. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. An effective immunization protocol for rabbits can be found in Vaitukaitis, J. et al. **J. Clin. Endocrinol. Metab. 33**:988-991 (1971).

**[0421]** Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. et al., Chap. 19 in: **Handbook of Experimental Immunology** D. Wier (ed) Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 μM). Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: **Manual of Clinical Immunology**, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D.C. (1980).

**[0422]** Antibody preparations prepared according to either protocol are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample. The antibodies may also be used in therapeutic compositions for killing cells expressing the protein or reducing the levels of the protein in the body.

## V. Use of Extended cDNAs or Portions Thereof as Reagents

**[0423]** The extended cDNAs of the present invention may be used as reagents in isolation procedures, diagnostic assays, and forensic procedures. For example, sequences from the extended cDNAs (or genomic DNAs obtainable therefrom) may be detectably labeled and used as probes to isolate other sequences capable of hybridizing to them. In addition, sequences from the extended cDNAs (or genomic DNAs obtainable therefrom) may be used to design PCR primers to be used in isolation, diagnostic, or forensic procedures.

## EXAMPLE 41

Preparation of PCR Primers and Amplification of DNA

**[0424]** The extended cDNAs (or genomic DNAs obtainable therefrom) may be used to prepare PCR primers for a variety of applications, including isolation procedures for cloning nucleic acids capable of hybridizing to such sequences, diagnostic techniques and forensic techniques. The PCR primers are at least 10 bases, and preferably at least 12, 15, or 17 bases in length. More preferably, the PCR primers are at least 20-30 bases in length. In some embodiments, the PCR primers may be more than 30 bases in length. It is preferred that the primer pairs have approximately the same G/C ratio, so that melting temperatures are approximately the same. A variety of PCR techniques are familiar to those skilled in the art. For a review of PCR technology, see Molecular Cloning to Genetic Engineering White, B.A. Ed. in Methods in Molecular Biology 67: Humana Press, Totowa 1997. In each of these PCR procedures, PCR primers on either side of the nucleic acid sequences to be amplified are added to a suitably prepared nucleic acid sample along with dNTPs and a thermostable polymerase such as Taq polymerase, Pfu polymerase, or Vent polymerase. The nucleic acid in the sample is denatured and the PCR primers are specifically hybridized to complementary nucleic acid sequences in the sample. The hybridized primers are extended. Thereafter, another cycle of denaturation, hybridization, and extension is initiated. The cycles are repeated multiple times to produce an amplified fragment containing the nucleic acid sequence between the primer sites.



## EXAMPLE 42

Use of Extended cDNAs as Probes

**[0425]** Probes derived from extended cDNAs or portions thereof (or genomic DNAs obtainable therefrom) may be labeled with detectable labels familiar to those skilled in the art, including radioisotopes and non-radioactive labels, to provide a detectable probe. The detectable probe may be single stranded or double stranded and may be made using techniques known in the art, including in vitro transcription, nick translation, or kinase reactions. A nucleic acid sample containing a sequence capable of

hybridizing to the labeled probe is contacted with the labeled probe. If the nucleic acid in the sample is double stranded, it may be denatured prior to contacting the probe. In some applications, the nucleic acid sample may be immobilized on a surface such as a nitrocellulose or nylon membrane. The nucleic acid sample may comprise nucleic acids obtained from a variety of sources, including genomic DNA, cDNA libraries, RNA, or tissue samples.

**[0426]** Procedures used to detect the presence of nucleic acids capable of hybridizing to the detectable probe include well known techniques such as Southern blotting, Northern blotting, dot blotting, colony hybridization, and plaque hybridization. In some applications, the nucleic acid capable of hybridizing to the labeled probe may be cloned into vectors such as expression vectors, sequencing vectors, or in vitro transcription vectors to facilitate the characterization and expression of the hybridizing nucleic acids in the sample. For example, such techniques may be used to isolate and clone sequences in a genomic library or cDNA library which are capable of hybridizing to the detectable probe as described in Example 30 above.

**[0427]** PCR primers made as described in Example 41 above may be used in forensic analyses, such as the DNA fingerprinting techniques described in Examples 43-47 below. Such analyses may utilize detectable probes or primers based on the sequences of the extended cDNAs isolated using the 5′ ESTs (or genomic DNAs obtainable therefrom).



## EXAMPLE 43

Forensic Matching by DNA Sequencing

**[0428]** In one exemplary method, DNA samples are isolated from forensic specimens of, for example, hair, semen, blood or skin cells by conventional methods. A panel of PCR primers based on a number of the extended cDNAs (or genomic DNAs obtainable therefrom), is then utilized in accordance with Example 41 to amplify DNA of approximately 100-200 bases in length from the forensic specimen. Corresponding sequences are obtained from a test subject. Each of these identification DNAs is then sequenced using standard techniques, and a simple database comparison determines the

differences, if any, between the sequences from the subject and those from the sample. Statistically significant differences between the suspect's DNA sequences and those from the sample conclusively prove a lack of identity. This lack of identity can be proven, for example, with only one sequence. Identity, on the other hand, should be demonstrated with a large number of sequences, all matching. Preferably, a minimum of 50 statistically identical sequences of 100 bases in length are used to prove identity between the suspect and the sample.

## EXAMPLE 44

Positive Identification by DNA Sequencing

**[0429]** The technique outlined in the previous example may also be used on a larger scale to provide a unique fingerprint-type identification of any individual. In this technique, primers are prepared from a large number of sequences from Table IV and the appended sequence listing. Preferably, 20 to 50 different primers are used. These primers are used to obtain a corresponding number of PCR-generated DNA segments from the individual in question in accordance with Example 41. Each of these DNA segments is sequenced, using the methods set forth in Example 43. The database of sequences generated through this procedure uniquely identifies the individual from whom the sequences were obtained. The same panel of primers may then be used at any later time to absolutely correlate tissue or other biological specimen with that individual.



## EXAMPLE 45

Southern Blot Forensic Identification

**[0430]** The procedure of Example 44 is repeated to obtain a panel of at least 10 amplified sequences from an individual and a specimen. Preferably, the panel contains at least 50 amplified sequences. More preferably, the panel contains 100 amplified sequences. In some embodiments, the panel contains 200 amplified sequences. This PCR-generated DNA is then digested with one or a combination of, preferably, four base specific restriction enzymes. Such enzymes are commercially available and known to

those of skill in the art. After digestion, the resultant gene fragments are size separated in multiple duplicate wells on an agarose gel and transferred to nitrocellulose using Southern blotting techniques well known to those with skill in the art. For a review of Southern blotting see Davis et al. (**Basic Methods in Molecular Biology**, 1986, Elsevier Press. pp 62-65).

**[0431]** A panel of probes based on the sequences of the extended cDNAs (or genomic DNAs obtainable therefrom), or fragments thereof of at least 10 bases, are radioactively or colorimetrically labeled using methods known in the art, such as nick translation or end labeling, and hybridized to the Southern blot using techniques known in the art (Davis et al., supra). Preferably, the probe comprises at least 12, 15, or 17 consecutive nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom). More preferably, the probe comprises at least 20-30 consecutive nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom). In some embodiments, the probe comprises more than 30 nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom). In other embodiments, the probe comprises at least 40, at least 50, at least 75, at least 100, at least 150, or at least 200 consecutive nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom).

**[0432]** Preferably, at least 5 to 10 of these labeled probes are used, and more preferably at least about 20 or 30 are used to provide a unique pattern. The resultant bands appearing from the hybridization of a large sample of extended cDNAs (or genomic DNAs obtainable therefrom) will be a unique identifier. Since the restriction enzyme cleavage will be different for every individual, the band pattern on the Southern blot will also be unique. Increasing the number of extended cDNA probes will provide a statistically higher level of confidence in the identification since there will be an increased number of sets of bands used for identification.

## EXAMPLE 46

Dot Blot Identification Procedure

**[0433]** Another technique for identifying individuals using the extended cDNA sequences disclosed herein utilizes a dot blot hybridization technique.

**[0434]** Genomic DNA is isolated from nuclei of subject to be identified. Oligonucleotide probes of approximately 30 bp in length are synthesized that correspond to at least 10, preferably 50 sequences from the extended cDNAs or genomic DNAs obtainable therefrom. The probes are used to hybridize to the genomic DNA through conditions known to those in the art. The oligonucleotides are end labeled with $P^{32}$ using polynucleotide kinase (Pharmacia). Dot Blots are created by spotting the genomic DNA onto nitrocellulose or the like using a vacuum dot blot manifold (BioRad, Richmond California). The nitrocellulose filter containing the genomic sequences is baked or UV linked to the filter, prehybridized and hybridized with labeled probe using techniques known in the art (Davis et al. supra). The $^{32}P$ labeled DNA fragments are sequentially hybridized with successively stringent conditions to detect minimal differences between the 30 bp sequence and the DNA. Tetramethylammonium chloride is useful for identifying clones containing small numbers of nucleotide mismatches (Wood et al., **Proc. Natl. Acad. Sci. USA 82(6)**:1585-1588 (1985)) which is hereby incorporated by reference. A unique pattern of dots distinguishes one individual from another individual.

**[0435]** Extended cDNAs or oligonucleotides containing at least 10 consecutive bases from these sequences can be used as probes in the following alternative fingerprinting technique. Preferably, the probe comprises at least 12, 15, or 17 consecutive nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom). More preferably, the probe comprises at least 20-30 consecutive nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom). In some embodiments, the probe comprises more than 30 nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom). In other embodiments, the probe comprises at least 40, at least 50, at least 75, at least 100, at least 150, or at least 200 consecutive nucleotides from the extended cDNA (or genomic DNAs obtainable therefrom).

**[0436]** Preferably, a plurality of probes having sequences from different genes are used in the alternative fingerprinting technique. Example 47 below provides a representative alternative fingerprinting procedure in which the probes are derived from extended cDNAs.

## EXAMPLE 47

Alternative "Fingerprint" Identification Technique

**[0437]** 20-mer oligonucleotides are prepared from a large number, e.g. 50, 100, or 200, of extended cDNA sequences (or genomic DNAs obtainable therefrom) using commercially available oligonucleotide services such as Genset, Paris, France. Cell samples from the test subject are processed for DNA using techniques well known to those with skill in the art. The nucleic acid is digested with restriction enzymes such as EcoRI and XbaI. Following digestion, samples are applied to wells for electrophoresis. The procedure, as known in the art, may be modified to accommodate polyacrylamide electrophoresis, however in this example, samples containing 5 ug of DNA are loaded into wells and separated on 0.8% agarose gels. The gels are transferred onto nitrocellulose using standard Southern blotting techniques.

**[0438]** 10 ng of each of the oligonucleotides are pooled and end-labeled with $P^{32}$. The nitrocellulose is prehybridized with blocking solution and hybridized with the labeled probes. Following hybridization and washing, the nitrocellulose filter is exposed to X-Omat AR X-ray film. The resulting hybridization pattern will be unique for each individual.

**[0439]** It is additionally contemplated within this example that the number of probe sequences used can be varied for additional accuracy or clarity.

**[0440]** The antibodies generated in Examples 30 and 40 above may be used to identify the tissue type or cell species from which a sample is derived as described above.

## EXAMPLE 48

Identification of Tissue Types or Cell Species by Means of

Labeled Tissue Specific Antibodies

**[0441]** Identification of specific tissues is accomplished by the visualization of tissue specific antigens by means of antibody preparations according to Examples 30 and 40 which are conjugated, directly or indirectly to a detectable marker. Selected labeled antibody species bind to their specific antigen binding partner in tissue sections, cell suspensions, or in extracts of soluble proteins from a tissue sample to provide a pattern for qualitative or semi-qualitative interpretation.

**[0442]** Antisera for these procedures must have a potency exceeding that of the native preparation, and for that reason, antibodies are concentrated to a mg/ml level by isolation of the gamma globulin fraction, for example, by ion-exchange chromatography or by ammonium sulfate fractionation. Also, to provide the most specific antisera, unwanted antibodies, for example to common proteins, must be removed from the gamma globulin fraction, for example by means of insoluble immunoabsorbents, before the antibodies are labeled with the marker. Either monoclonal or heterologous antisera is suitable for either procedure.

### A. Immunohistochemical Techniques

**[0443]** Purified, high-titer antibodies, prepared as described above, are conjugated to a detectable marker, as described, for example, by Fudenberg, H., Chap. 26 in: **Basic 503 Clinical Immunology**, 3rd Ed. Lange, Los Altos, California (1980) or Rose, N. et al., Chap. 12 in: **Methods in Immunodiagnosis**, 2d Ed. John Wiley 503 Sons, New York (1980).

**[0444]** A fluorescent marker, either fluorescein or rhodamine, is preferred, but antibodies can also be labeled with an enzyme that supports a color producing reaction with a substrate, such as horseradish peroxidase. Markers can be added to tissue-bound antibody in a second step, as described below. Alternatively, the specific antitissue antibodies can be labeled with ferritin or other electron dense particles, and localization of the ferritin coupled antigen-antibody complexes achieved by means of an electron microscope. In yet another approach, the antibodies are radiolabeled, with, for example

$^{125}I$, and detected by overlaying the antibody treated preparation with photographic emulsion.

**[0445]** Preparations to carry out the procedures can comprise monoclonal or polyclonal antibodies to a single protein or peptide identified as specific to a tissue type, for example, brain tissue, or antibody preparations to several antigenically distinct tissue specific antigens can be used in panels, independently or in mixtures, as required.

**[0446]** Tissue sections and cell suspensions are prepared for immunohistochemical examination according to common histological techniques. Multiple cryostat sections (about 4 μm, unfixed) of the unknown tissue and known control, are mounted and each slide covered with different dilutions of the antibody preparation. Sections of known and unknown tissues should also be treated with preparations to provide a positive control, a negative control, for example, pre-immune sera, and a control for non-specific staining, for example, buffer.

**[0447]** Treated sections are incubated in a humid chamber for 30 min at room temperature, rinsed, then washed in buffer for 30-45 min. Excess fluid is blotted away, and the marker developed.

**[0448]** If the tissue specific antibody was not labeled in the first incubation, it can be labeled at this time in a second antibody-antibody reaction, for example, by adding fluorescein- or enzyme-conjugated antibody against the immunoglobulin class of the antiserum-producing species, for example, fluorescein labeled antibody to mouse IgG. Such labeled sera are commercially available.

**[0449]** The antigen found in the tissues by the above procedure can be quantified by measuring the intensity of color or fluorescence on the tissue section, and calibrating that signal using appropriate standards.

**B. Identification of Tissue Specific Soluble Proteins**

**[0450]** The visualization of tissue specific proteins and identification of unknown tissues from that procedure is carried out using the labeled antibody reagents and detection strategy as described for immunohistochemistry; however the sample is prepared according to an electrophoretic technique to distribute the proteins extracted from the tissue in an orderly array on the basis of molecular weight for detection.

**[0451]** A tissue sample is homogenized using a Virtis apparatus; cell suspensions are disrupted by Dounce homogenization or osmotic lysis, using detergents in either case as required to disrupt cell membranes, as is the practice in the art. Insoluble cell components such as nuclei, microsomes, and membrane fragments are removed by ultracentrifugation, and the soluble protein-containing fraction concentrated if necessary and reserved for analysis.

**[0452]** A sample of the soluble protein solution is resolved into individual protein species by conventional SDS polyacrylamide electrophoresis as described, for example, by Davis, L. et al., Section 19-2 in: **Basic Methods in Molecular Biology** (P. Leder, ed), Elsevier, New York (1986), using a range of amounts of polyacrylamide in a set of gels to resolve the entire molecular weight range of proteins to be detected in the sample. A size marker is run in parallel for purposes of estimating molecular weights of the constituent proteins. Sample size for analysis is a convenient volume of from 5 to55 µl, and containing from about 1 to 100 µg protein. An aliquot of each of the resolved proteins is transferred by blotting to a nitrocellulose filter paper, a process that maintains the pattern of resolution. Multiple copies are prepared. The procedure, known as Western Blot Analysis, is well described in Davis, L. et al., (above) Section 19-3. One set of nitrocellulose blots is stained with Coomassie Blue dye to visualize the entire set of proteins for comparison with the antibody bound proteins. The remaining nitrocellulose filters are then incubated with a solution of one or more specific antisera to tissue specific proteins prepared as described in Examples 30 and 40. In this procedure, as in procedure A above, appropriate positive and negative sample and reagent controls are run.

**[0453]** In either procedure A or B, a detectable label can be attached to the primary tissue antigen-primary antibody complex according to various strategies and permutations thereof. In a straightforward approach, the primary specific antibody can be labeled; alternatively, the unlabeled complex can be bound by a labeled secondary anti-IgG antibody. In other approaches, either the primary or secondary antibody is conjugated to a biotin molecule, which can, in a subsequent step, bind an avidin conjugated marker. According to yet another strategy, enzyme labeled or radioactive protein A, which has the property of binding to any IgG, is bound in a final step to either the primary or secondary antibody.

**[0454]** The visualization of tissue specific antigen binding at levels above those seen in control tissues to one or more tissue specific antibodies, prepared from the gene sequences identified from extended cDNA sequences, can identify tissues of unknown origin, for example, forensic samples, or differentiated tumor tissue that has metastasized to foreign bodily sites.

**[0455]** In addition to their applications in forensics and identification, extended cDNAs (or genomic DNAs obtainable therefrom) may be mapped to their chromosomal locations. Example 49 below describes radiation hybrid (RH) mapping of human chromosomal regions using extended cDNAs. Example 50 below describes a representative procedure for mapping an extended cDNA (or a genomic DNA obtainable therefrom) to its location on a human chromosome. Example 51 below describes mapping of extended cDNAs (or genomic DNAs obtainable therefrom) on metaphase chromosomes by Fluorescence In Situ Hybridization (FISH).

## EXAMPLE 49

Radiation hybrid mapping of Extended cDNAs to the human genome

**[0456]** Radiation hybrid (RH) mapping is a somatic cell genetic approach that can be used for high resolution mapping of the human genome. In this approach, cell lines containing one or more human chromosomes are lethally irradiated, breaking each chromosome into fragments whose size depends on the radiation dose. These fragments are rescued by fusion with cultured rodent cells, yielding subclones containing different portions of the human genome. This technique is described by Benham et al. (*Genomics* **4**:509-517, 1989) and Cox et al., (*Science* **250**:245-250, 1990), the entire contents of which are hereby incorporated by reference. The random and independent nature of the subclones permits efficient mapping of any human genome marker. Human DNA isolated from a panel of 80-100 cell lines provides a mapping reagent for ordering extended cDNAs (or genomic DNAs obtainable therefrom). In this approach, the frequency of breakage between markers is used to measure distance, allowing construction of fine resolution maps as has been done using conventional ESTs (Schuler et al., *Science* **274**:540-546, 1996, hereby incorporated by reference).

**[0457]** RH mapping has been used to generate a high-resolution whole genome radiation hybrid map of human chromosome 17q22-q25.3 across the genes for growth hormone (GH) and thymidine kinase (TK) (Foster et al., *Genomics* **33**:185-192, 1996), the region surrounding the Gorlin syndrome gene (Obermayr et al., *Eur. J. Hum. Genet.* **4**:242-245, 1996), 60 loci covering the entire short arm of chromosome 12 (Raeymaekers et al., *Genomics* **29**:170-178, 1995), the region of human chromosome 22 containing the neurofibromatosis type 2 locus (Frazer et al., *Genomics* **14**:574-584, 1992) and 13 loci on the long arm of chromosome 5 (Warrington et al., *Genomics* **11**:701-708, 1991).

## EXAMPLE 50

Mapping of Extended cDNAs to Human

Chromosomes using PCR techniques

**[0458]** Extended cDNAs (or genomic DNAs obtainable therefrom) may be assigned to human chromosomes using PCR based methodologies. In such approaches, oligonucleotide primer pairs are designed from the extended cDNA sequence (or the sequence of a genomic DNA obtainable therefrom) to minimize the chance of amplifying through an intron. Preferably, the oligonucleotide primers are 18-23 bp in length and are designed for PCR amplification. The creation of PCR primers from known sequences is well known to those with skill in the art. For a review of PCR technology see Erlich, H.A., **PCR Technology; Principles and Applications for DNA Amplification**, 1992. W.H. Freeman and Co., New York.

**[0459]** The primers are used in polymerase chain reactions (PCR) to amplify templates from total human genomic DNA. PCR conditions are as follows: 60 ng of genomic DNA is used as a template for PCR with 80 ng of each oligonucleotide primer, 0.6 unit of Taq polymerase, and 1 μCu of a $^{32}$P-labeled deoxycytidine triphosphate. The PCR is performed in a microplate thermocycler (Techne) under the following conditions: 30 cycles of 94°C, 1.4 min; 55°C, 2 min; and 72°C, 2 min; with a final extension at 72°C for 10 min. The amplified products are analyzed on a 6% polyacrylamide sequencing gel and visualized by autoradiography. If the length of the resulting PCR product is identical to the distance between the ends of the primer sequences in the extended cDNA from which the primers are derived, then the PCR reaction is repeated with DNA templates from two panels of human-rodent somatic cell hybrids, BIOS PCRable DNA (BIOS Corporation) and NIGMS Human-Rodent Somatic Cell Hybrid Mapping Panel Number 1 (NIGMS, Camden, NJ).

**[0460]** PCR is used to screen a series of somatic cell hybrid cell lines containing defined sets of human chromosomes for the presence of a given extended cDNA (or genomic DNA obtainable therefrom). DNA is isolated from the somatic hybrids and used as starting templates for PCR reactions using the primer pairs from the extended cDNAs (or genomic DNAs obtainable therefrom). Only those somatic cell

hybrids with chromosomes containing the human gene corresponding to the extended cDNA (or genomic DNA obtainable therefrom) will yield an amplified fragment. The extended cDNAs (or genomic DNAs obtainable therefrom) are assigned to a chromosome by analysis of the segregation pattern of PCR products from the somatic hybrid DNA templates. The single human chromosome present in all cell hybrids that give rise to an amplified fragment is the chromosome containing that extended cDNA (or genomic DNA obtainable therefrom). For a review of techniques and analysis of results from somatic cell gene mapping experiments. (See Ledbetter et al., **Genomics** 6:475-481 (1990).)

**[0461]** Alternatively, the extended cDNAs (or genomic DNAs obtainable therefrom) may be mapped to individual chromosomes using FISH as described in Example 51 below.



## EXAMPLE 51

Mapping of Extended 5′ ESTs to Chromosomes

Using Fluorescence in situ Hybridization

**[0462]** Fluorescence in situ hybridization allows the extended cDNA (or genomic DNA obtainable therefrom) to be mapped to a particular location on a given chromosome. The chromosomes to be used for fluorescence in situ hybridization techniques may be obtained from a variety of sources including cell cultures, tissues, or whole blood.

**[0463]** In a preferred embodiment, chromosomal localization of an extended cDNA (or genomic DNA obtainable therefrom) is obtained by FISH as described by Cherif et al. (*Proc. Natl. Acad. Sci. U.S.A.*, **87**:6639-6643, 1990). Metaphase chromosomes are prepared from phytohemagglutinin (PHA)-stimulated blood cell donors. PHA-stimulated lymphocytes from healthy males are cultured for 72 h in RPMI-1640 medium. For synchronization, methotrexate (10 μM) is added for 17 h, followed by addition of 5-bromodeoxyuridine (5-BudR, 0.1 mM) for 6 h. Colcemid (1 μg/ml) is added for the last 15 min before harvesting the cells. Cells are collected, washed in RPMI, incubated with a hypotonic solution of KCl (75 mM) at 37°C for 15 min and fixed in three

changes of methanol:acetic acid (3:1). The cell suspension is dropped onto a glass slide and air dried. The extended cDNA (or genomic DNA obtainable therefrom) is labeled with biotin-16 dUTP by nick translation according to the manufacturer's instructions (Bethesda Research Laboratories, Bethesda, MD), purified using a Sephadex G-50 column (Pharmacia, Upssala, Sweden) and precipitated. Just prior to hybridization, the DNA pellet is dissolved in hybridization buffer (50% formamide, 2 X SSC, 10% dextran sulfate, 1 mg/ml sonicated salmon sperm DNA, pH 7) and the probe is denatured at 70°C for 5-10 min.

**[0464]** Slides kept at -20°C are treated for 1 h at 37°C with RNase A (100 μg/ml), rinsed three times in 2 X SSC and dehydrated in an ethanol series. Chromosome preparations are denatured in 70% formamide, 2 X SSC for 2 min at 70°C, then dehydrated at 4°C. The slides are treated with proteinase K (10 μg/100 ml in 20 mM Tris-HCl, 2 mM $CaCl_2$) at 37°C for 8 min and dehydrated. The hybridization mixture containing the probe is placed on the slide, covered with a coverslip, sealed with rubber cement and incubated overnight in a humid chamber at 37°C. After hybridization and post-hybridization washes, the biotinylated probe is detected by avidin-FITC and amplified with additional layers of biotinylated goat anti-avidin and avidin-FITC. For chromosomal localization, fluorescent R-bands are obtained as previously described (Cherif et al., *supra.*). The slides are observed under a LEICA fluorescence microscope (DMRXA). Chromosomes are counterstained with propidium iodide and the fluorescent signal of the probe appears as two symmetrical yellow-green spots on both chromatids of the fluorescent R-band chromosome (red). Thus, a particular extended cDNA (or genomic DNA obtainable therefrom) may be localized to a particular cytogenetic R-band on a given chromosome.

**[0465]** Once the extended cDNAs (or genomic DNAs obtainable therefrom) have been assigned to particular chromosomes using the techniques described in Examples 49-51 above, they may be utilized to construct a high resolution map of the chromosomes on which they are located or to identify the chromosomes in a sample.

## EXAMPLE 52

Use of Extended cDNAs to Construct or Expand Chromosome Maps

**[0466]** Chromosome mapping involves assigning a given unique sequence to a particular chromosome as described above. Once the unique sequence has been mapped to a given chromosome, it is ordered relative to other unique sequences located on the same chromosome. One approach to chromosome mapping utilizes a series of yeast artificial chromosomes (YACs) bearing several thousand long inserts derived from the chromosomes of the organism from which the extended cDNAs (or genomic DNAs obtainable therefrom) are obtained. This approach is described in Ramaiah Nagaraja et al. **Genome Research** 7:210-222, March 1997. Briefly, in this approach each chromosome is broken into overlapping pieces which are inserted into the YAC vector. The YAC inserts are screened using PCR or other methods to determine whether they include the extended cDNA (or genomic DNA obtainable therefrom) whose position is to be determined. Once an insert has been found which includes the extended cDNA (or genomic DNA obtainable therefrom), the insert can be analyzed by PCR or other methods to determine whether the insert also contains other sequences known to be on the chromosome or in the region from which the extended cDNA (or genomic DNA obtainable therefrom) was derived. This process can be repeated for each insert in the YAC library to determine the location of each of the extended cDNAs (or genomic DNAs obtainable therefrom) relative to one another and to other known chromosomal markers. In this way, a high resolution map of the distribution of numerous unique markers along each of the organisms chromosomes may be obtained.

**[0467]** As described in Example 53 below extended cDNAs (or genomic DNAs obtainable therefrom) may also be used to identify genes associated with a particular phenotype, such as hereditary disease or drug response.

## EXAMPLE 53

Identification of genes associated with hereditary diseases or drug response

**[0468]** This example illustrates an approach useful for the association of extended cDNAs (or genomic DNAs obtainable therefrom) with particular phenotypic

characteristics. In this example, a particular extended cDNA (or genomic DNA obtainable therefrom) is used as a test probe to associate that extended cDNA (or genomic DNA obtainable therefrom) with a particular phenotypic characteristic.

**[0469]** Extended cDNAs (or genomic DNAs obtainable therefrom) are mapped to a particular location on a human chromosome using techniques such as those described in Examples 49 and 50 or other techniques known in the art. A search of Mendelian Inheritance in Man (V. McKusick, **Mendelian Inheritance in Man** (available on line through Johns Hopkins University Welch Medical Library) reveals the region of the human chromosome which contains the extended cDNA (or genomic DNA obtainable therefrom) to be a very gene rich region containing several known genes and several diseases or phenotypes for which genes have not been identified. The gene corresponding to this extended cDNA (or genomic DNA obtainable therefrom) thus becomes an immediate candidate for each of these genetic diseases.

**[0470]** Cells from patients with these diseases or phenotypes are isolated and expanded in culture. PCR primers from the extended cDNA (or genomic DNA obtainable therefrom) are used to screen genomic DNA, mRNA or cDNA obtained from the patients. Extended cDNAs (or genomic DNAs obtainable therefrom) that are not amplified in the patients can be positively associated with a particular disease by further analysis. Alternatively, the PCR analysis may yield fragments of different lengths when the samples are derived from an individual having the phenotype associated with the disease than when the sample is derived from a healthy individual, indicating that the gene containing the extended cDNA may be responsible for the genetic disease.

## VI. Use of Extended cDNAs (or genomic DNAs obtainable therefrom) to Construct Vectors

**[0471]** The present extended cDNAs (or genomic DNAs obtainable therefrom) may also be used to construct secretion vectors capable of directing the secretion of the proteins encoded by genes inserted in the vectors. Such secretion vectors may facilitate the purification or enrichment of the proteins encoded by genes inserted therein by reducing the number of background proteins from which the desired protein must be purified or enriched. Exemplary secretion vectors are described in Example 54 below.

## EXAMPLE 54

Construction of Secretion Vectors

**[0472]** The secretion vectors of the present invention include a promoter capable of directing gene expression in the host cell, tissue, or organism of interest. Such promoters include the Rous Sarcoma Virus promoter, the SV40 promoter, the human cytomegalovirus promoter, and other promoters familiar to those skilled in the art.

**[0473]** A signal sequence from an extended cDNA (or genomic DNA obtainable therefrom), such as one of the signal sequences in SEQ ID NOs: 40-59, 61-73, 75-82, 84, and 130-154 as defined in Table IV above, is operably linked to the promoter such that the mRNA transcribed from the promoter will direct the translation of the signal peptide. The host cell, tissue, or organism may be any cell, tissue, or organism which recognizes the signal peptide encoded by the signal sequence in the extended cDNA (or genomic DNA obtainable therefrom). Suitable hosts include mammalian cells, tissues or organisms, avian cells, tissues, or organisms, insect cells, tissues or organisms, or yeast.

**[0474]** In addition, the secretion vector contains cloning sites for inserting genes encoding the proteins which are to be secreted. The cloning sites facilitate the cloning of the insert gene in frame with the signal sequence such that a fusion protein in which the signal peptide is fused to the protein encoded by the inserted gene is expressed from the mRNA transcribed from the promoter. The signal peptide directs the extracellular secretion of the fusion protein.

**[0475]** The secretion vector may be DNA or RNA and may integrate into the chromosome of the host, be stably maintained as an extrachromosomal replicon in the host, be an artificial chromosome, or be transiently present in the host. Many nucleic acid backbones suitable for use as secretion vectors are known to those skilled in the art, including retroviral vectors, SV40 vectors, Bovine Papilloma Virus vectors, yeast integrating plasmids, yeast episomal plasmids, yeast artificial chromosomes, human artificial chromosomes, P element vectors, baculovirus vectors, or bacterial plasmids capable of being transiently introduced into the host.

**[0476]** The secretion vector may also contain a polyA signal such that the polyA signal is located downstream of the gene inserted into the secretion vector.

**[0477]** After the gene encoding the protein for which secretion is desired is inserted into the secretion vector, the secretion vector is introduced into the host cell, tissue, or organism using calcium phosphate precipitation, DEAE-Dextran, electroporation, liposome-mediated transfection, viral particles or as naked DNA. The protein encoded by the inserted gene is then purified or enriched from the supernatant using conventional techniques such as ammonium sulfate precipitation, immunoprecipitation, immunochromatography, size exclusion chromatography, ion exchange chromatography, and hplc. Alternatively, the secreted protein may be in a sufficiently enriched or pure state in the supernatant or growth media of the host to permit it to be used for its intended purpose without further enrichment.

**[0478]** The signal sequences may also be inserted into vectors designed for gene therapy. In such vectors, the signal sequence is operably linked to a promoter such that mRNA transcribed from the promoter encodes the signal peptide. A cloning site is located downstream of the signal sequence such that a gene encoding a protein whose secretion is desired may readily be inserted into the vector and fused to the signal sequence. The vector is introduced into an appropriate host cell. The protein expressed from the promoter is secreted extracellularly, thereby producing a therapeutic effect.

**[0479]** The extended cDNAs or 5′ ESTs may also be used to clone sequences located upstream of the extended cDNAs or 5′ ESTs which are capable of regulating gene expression, including promoter sequences, enhancer sequences, and other upstream sequences which influence transcription or translation levels. Once identified and cloned, these upstream regulatory sequences may be used in expression vectors designed to direct the expression of an inserted gene in a desired spatial, temporal, developmental, or quantitative fashion. Example 55 describes a method for cloning sequences upstream of the extended cDNAs or 5′ ESTs.

## EXAMPLE 55

Use of Extended cDNAs or 5′ ESTs to Clone Upstream

Sequences from Genomic DNA

**[0480]** Sequences derived from extended cDNAs or 5′ ESTs may be used to isolate the promoters of the corresponding genes using chromosome walking techniques. In one chromosome walking technique, which utilizes the GenomeWalker™ kit available from Clontech, five complete genomic DNA samples are each digested with a different restriction enzyme which has a 6 base recognition site and leaves a blunt end. Following digestion, oligonucleotide adapters are ligated to each end of the resulting genomic DNA fragments.

**[0481]** For each of the five genomic DNA libraries, a first PCR reaction is performed according to the manufacturer's instructions (which are incorporated herein by reference) using an outer adaptor primer provided in the kit and an outer gene specific primer. The gene specific primer should be selected to be specific for the extended cDNA or 5′ EST of interest and should have a melting temperature, length, and location in the extended cDNA or ′ EST which is consistent with its use in PCR reactions. Each first PCR reaction contains 5ng of genomic DNA, 5 µl of 10X Tth reaction buffer, 0.2 mM of each dNTP, 0.2 µM each of outer adaptor primer and outer gene specific primer, 1.1 mM of $Mg(OAc)_2$, and 1 µl of the Tth polymerase 50X mix in a total volume of 50 µl. The reaction cycle for the first PCR reaction is as follows: 1 min @ 94°C / 2 sec @ 94°C, 3 min @ 72°C (7 cycles) / 2 sec @ 94°C, 3 min @ 67°C (32 cycles) / 5 min @ 67°C.

**[0482]** The product of the first PCR reaction is diluted and used as a template for a second PCR reaction according to the manufacturer's instructions using a pair of nested primers which are located internally on the amplicon resulting from the first PCR reaction. For example, 5 µl of the reaction product of the first PCR reaction mixture may be diluted 180 times. Reactions are made in a 50 µl volume having a composition identical to that of the first PCR reaction except the nested primers are used. The first nested primer is specific for the adaptor, and is provided with the GenomeWalker™ kit. The second nested primer is specific for the particular extended cDNA or 5′ EST for which the promoter is to be cloned and should have a melting temperature, length, and location in

the extended cDNA or 5′ EST which is consistent with its use in PCR reactions. The reaction parameters of the second PCR reaction are as follows: 1 min @ 94°C / 2 sec @ 94°C, 3 min @ 72°C (6 cycles) / 2 sec @ 94°C, 3 min @ 67°C (25 cycles) / 5 min @ 67°C.

**[0483]** The product of the second PCR reaction is purified, cloned, and sequenced using standard techniques. Alternatively, two or more human genomic DNA libraries can be constructed by using two or more restriction enzymes. The digested genomic DNA is cloned into vectors which can be converted into single stranded, circular, or linear DNA. A biotinylated oligonucleotide comprising at least 15 nucleotides from the extended cDNA or 5′ EST sequence is hybridized to the single stranded DNA. Hybrids between the biotinylated oligonucleotide and the single stranded DNA containing the extended cDNA or EST sequence are isolated as described in Example 29 above. Thereafter, the single stranded DNA containing the extended cDNA or EST sequence is released from the beads and converted into double stranded DNA using a primer specific for the extended cDNA or 5′ EST sequence or a primer corresponding to a sequence included in the cloning vector. The resulting double stranded DNA is transformed into bacteria. DNAs containing the 5′ EST or extended cDNA sequences are identified by colony PCR or colony hybridization.

**[0484]** Once the upstream genomic sequences have been cloned and sequenced as described above, prospective promoters and transcription start sites within the upstream sequences may be identified by comparing the sequences upstream of the extended cDNAs or 5′ ESTs with databases containing known transcription start sites, transcription factor binding sites, or promoter sequences.

**[0485]** In addition, promoters in the upstream sequences may be identified using promoter reporter vectors as described in Example 56.

## EXAMPLE 56

Identification of Promoters in Cloned Upstream Sequences

**[0486]** The genomic sequences upstream of the extended cDNAs or 5′ ESTs are cloned into a suitable promoter reporter vector, such as the pSEAP-Basic, pSEAP-Enhancer, pβgal-Basic, pβgal-Enhancer, or pEGFP-1 Promoter Reporter vectors available from Clontech. Briefly, each of these promoter reporter vectors include multiple cloning sites positioned upstream of a reporter gene encoding a readily assayable protein such as secreted alkaline phosphatase, β galactosidase, or green fluorescent protein. The sequences upstream of the extended cDNAs or 5′ ESTs are inserted into the cloning sites upstream of the reporter gene in both orientations and introduced into an appropriate host cell. The level of reporter protein is assayed and compared to the level obtained from a vector which lacks an insert in the cloning site. The presence of an elevated expression level in the vector containing the insert with respect to the control vector indicates the presence of a promoter in the insert. If necessary, the upstream sequences can be cloned into vectors which contain an enhancer for augmenting transcription levels from weak promoter sequences. A significant level of expression above that observed with the vector lacking an insert indicates that a promoter sequence is present in the inserted upstream sequence.

**[0487]** Appropriate host cells for the promoter reporter vectors may be chosen based on the results of the above described determination of expression patterns of the extended cDNAs and ESTs. For example, if the expression pattern analysis indicates that the mRNA corresponding to a particular extended cDNA or 5′ EST is expressed in fibroblasts, the promoter reporter vector may be introduced into a human fibroblast cell line.

**[0488]** Promoter sequences within the upstream genomic DNA may be further defined by constructing nested deletions in the upstream DNA using conventional techniques such as Exonuclease III digestion. The resulting deletion fragments can be inserted into the promoter reporter vector to determine whether the deletion has reduced or obliterated promoter activity. In this way, the boundaries of the promoters may be defined. If desired, potential individual regulatory sites within the promoter may be identified using

site directed mutagenesis or linker scanning to obliterate potential transcription factor binding sites within the promoter individually or in combination. The effects of these mutations on transcription levels may be determined by inserting the mutations into the cloning sites in the promoter reporter vectors.

## EXAMPLE 57

Cloning and Identification of Promoters

**[0489]** Using the method described in Example 55 above with 5′ ESTs, sequences upstream of several genes were obtained. Using the primer pairs GGG AAG ATG GAG ATA GTA TTG CCT G (SEQ ID NO:29) and CTG CCA TGT ACA TGA TAG AGA GAT TC (SEQ ID NO:30), the promoter having the internal designation P13H2 (SEQ ID NO:31) was obtained.

**[0490]** Using the primer pairs GTA CCA GGGG ACT GTG ACC ATT GC (SEQ ID NO:32) and CTG TGA CCA TTG CTC CCA AGA GAG (SEQ ID NO:33), the promoter having the internal designation P15B4 (SEQ ID NO:34) was obtained.

**[0491]** Using the primer pairs CTG GGA TGG AAG GCA CGG TA (SEQ ID NO:35) and GAG ACC ACA CAG CTA GAC AA (SEQ ID NO:36), the promoter having the internal designation P29B6 (SEQ ID NO:37) was obtained.

**[0492]** Figure 8 provides a schematic description of the promoters isolated and the way they are assembled with the corresponding 5′ tags. The upstream sequences were screened for the presence of motifs resembling transcription factor binding sites or known transcription start sites using the computer program MatInspector release 2.0, August 1996.

**[0493]** Figure 9 describes the transcription factor binding sites present in each of these promoters. The columns labeled matrice provides the name of the MatInspector matrix used. The column labeled position provides the 5′ position of the promoter site. Numeration of the sequence starts from the transcription site as determined by matching the genomic sequence with the 5′ EST sequence. The column labeled "orientation" indicates the DNA strand on which the site is found, with the + strand being

the coding strand as determined by matching the genomic sequence with the sequence of the 5′ EST. The column labeled "score" provides the MatInspector score found for this site. The column labeled "length" provides the length of the site in nucleotides. The column labeled "sequence" provides the sequence of the site found.

**[0494]** The promoters and other regulatory sequences located upstream of the extended cDNAs or 5′ ESTs may be used to design expression vectors capable of directing the expression of an inserted gene in a desired spatial, temporal, developmental, or quantitative manner. A promoter capable of directing the desired spatial, temporal, developmental, and quantitative patterns may be selected using the results of the expression analysis described in Example 26 above. For example, if a promoter which confers a high level of expression in muscle is desired, the promoter sequence upstream of an extended cDNA or 5′ EST derived from an mRNA which is expressed at a high level in muscle, as determined by the method of Example 26, may be used in the expression vector.

**[0495]** Preferably, the desired promoter is placed near multiple restriction sites to facilitate the cloning of the desired insert downstream of the promoter, such that the promoter is able to drive expression of the inserted gene. The promoter may be inserted in conventional nucleic acid backbones designed for extrachromosomal replication, integration into the host chromosomes or transient expression. Suitable backbones for the present expression vectors include retroviral backbones, backbones from eukaryotic episomes such as SV40 or Bovine Papilloma Virus, backbones from bacterial episomes, or artificial chromosomes.

**[0496]** Preferably, the expression vectors also include a polyA signal downstream of the multiple restriction sites for directing the polyadenylation of mRNA transcribed from the gene inserted into the expression vector.

**[0497]** Following the identification of promoter sequences using the procedures of Examples 55-57, proteins which interact with the promoter may be identified as described in Example 58 below.

## EXAMPLE 58

Identification of Proteins Which Interact with Promoter Sequences, Upstream Regulatory Sequences, or mRNA

**[0498]** Sequences within the promoter region which are likely to bind transcription factors may be identified by homology to known transcription factor binding sites or through conventional mutagenesis or deletion analyses of reporter plasmids containing the promoter sequence. For example, deletions may be made in a reporter plasmid containing the promoter sequence of interest operably linked to an assayable reporter gene. The reporter plasmids carrying various deletions within the promoter region are transfected into an appropriate host cell and the effects of the deletions on expression levels is assessed. Transcription factor binding sites within the regions in which deletions reduce expression levels may be further localized using site directed mutagenesis, linker scanning analysis, or other techniques familiar to those skilled in the art. Nucleic acids encoding proteins which interact with sequences in the promoter may be identified using one-hybrid systems such as those described in the manual accompanying the Matchmaker One-Hybrid System kit available from Clontech (Catalog No. K1603-1), the disclosure of which is incorporated herein by reference. Briefly, the Matchmaker One-hybrid system is used as follows. The target sequence for which it is desired to identify binding proteins is cloned upstream of a selectable reporter gene and integrated into the yeast genome. Preferably, multiple copies of the target sequences are inserted into the reporter plasmid in tandem.

**[0499]** A library comprised of fusions between cDNAs to be evaluated for the ability to bind to the promoter and the activation domain of a yeast transcription factor, such as GAL4, is transformed into the yeast strain containing the integrated reporter sequence. The yeast are plated on selective media to select cells expressing the selectable marker linked to the promoter sequence. The colonies which grow on the selective media contain genes encoding proteins which bind the target sequence. The inserts in the genes encoding the fusion proteins are further characterized by sequencing. In addition, the inserts may be inserted into expression vectors or in vitro transcription vectors. Binding of the polypeptides encoded by the inserts to the promoter DNA may be confirmed by

techniques familiar to those skilled in the art, such as gel shift analysis or DNAse protection analysis.

## VII. Use of Extended cDNAs (or Genomic DNAs Obtainable Therefrom) in Gene Therapy

**[0500]** The present invention also comprises the use of extended cDNAs (or genomic DNAs obtainable therefrom) in gene therapy strategies, including antisense and triple helix strategies as described in Examples 57 and 58 below. In antisense approaches, nucleic acid sequences complementary to an mRNA are hybridized to the mRNA intracellularly, thereby blocking the expression of the protein encoded by the mRNA. The antisense sequences may prevent gene expression through a variety of mechanisms. For example, the antisense sequences may inhibit the ability of ribosomes to translate the mRNA. Alternatively, the antisense sequences may block transport of the mRNA from the nucleus to the cytoplasm, thereby limiting the amount of mRNA available for translation. Another mechanism through which antisense sequences may inhibit gene expression is by interfering with mRNA splicing. In yet another strategy, the antisense nucleic acid may be incorporated in a ribozyme capable of specifically cleaving the target mRNA.

## EXAMPLE 59

Preparation and Use of Antisense Oligonucleotides

**[0501]** The antisense nucleic acid molecules to be used in gene therapy may be either DNA or RNA sequences. They may comprise a sequence complementary to the sequence of the extended cDNA (or genomic DNA obtainable therefrom). The antisense nucleic acids should have a length and melting temperature sufficient to permit formation of an intracellular duplex having sufficient stability to inhibit the expression of the mRNA in the duplex. Strategies for designing antisense nucleic acids suitable for use in gene therapy are disclosed in Green et al., **Ann. Rev. Biochem. 55**:569-597 (1986) and Izant and Weintraub, **Cell 36**:1007-1015 (1984), which are hereby incorporated by reference.

**[0502]** In some strategies, antisense molecules are obtained from a nucleotide sequence encoding a protein by reversing the orientation of the coding region with respect to a promoter so as to transcribe the opposite strand from that which is normally transcribed in the cell. The antisense molecules may be transcribed using in vitro transcription systems such as those which employ T7 or SP6 polymerase to generate the transcript. Another approach involves transcription of the antisense nucleic acids in vivo by operably linking DNA containing the antisense sequence to a promoter in an expression vector.

**[0503]** Alternatively, oligonucleotides which are complementary to the strand normally transcribed in the cell may be synthesized in vitro. Thus, the antisense nucleic acids are complementary to the corresponding mRNA and are capable of hybridizing to the mRNA to create a duplex. In some embodiments, the antisense sequences may contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of modifications suitable for use in antisense strategies are described by Rossi et al., **Pharmacol. Ther. 50(2)**:245-254, (1991).

**[0504]** Various types of antisense oligonucleotides complementary to the sequence of the extended cDNA (or genomic DNA obtainable therefrom) may be used. In one preferred embodiment, stable and semi-stable antisense oligonucleotides described in

International Application No. PCT WO94/23026, hereby incorporated by reference, are used. In these molecules, the 3′ end or both the 3′ and 5′ ends are engaged in intramolecular hydrogen bonding between complementary base pairs. These molecules are better able to withstand exonuclease attacks and exhibit increased stability compared to conventional antisense oligonucleotides.

**[0505]** In another preferred embodiment, the antisense oligodeoxynucleotides against herpes simplex virus types 1 and 2 described in International Application No. WO 95/04141, hereby incorporated by reference, are used.

**[0506]** In yet another preferred embodiment, the covalently cross-linked antisense oligonucleotides described in International Application No. WO 96/31523, hereby incorporated by reference, are used. These double- or single-stranded oligonucleotides comprise one or more, respectively, inter- or intra-oligonucleotide covalent cross-linkages, wherein the linkage consists of an amide bond between a primary amine group of one strand and a carboxyl group of the other strand or of the same strand, respectively, the primary amine group being directly substituted in the 2' position of the strand nucleotide monosaccharide ring, and the carboxyl group being carried by an aliphatic spacer group substituted on a nucleotide or nucleotide analog of the other strand or the same strand, respectively.

**[0507]** The antisense oligodeoxynucleotides and oligonucleotides disclosed in International Application No. WO 92/18522, incorporated by reference, may also be used. These molecules are stable to degradation and contain at least one transcription control recognition sequence which binds to control proteins and are effective as decoys therefor. These molecules may contain "hairpin" structures, "dumbbell" structures, "modified dumbbell" structures, "cross-linked" decoy structures and "loop" structures.

**[0508]** In another preferred embodiment, the cyclic double-stranded oligonucleotides described in European Patent Application No. 0 572 287 A2, hereby incorporated by reference are used. These ligated oligonucleotide "dumbbells" contain the binding site for a transcription factor and inhibit expression of the gene under control of the transcription factor by sequestering the factor.

**[0509]** Use of the closed antisense oligonucleotides disclosed in International Application No. WO 92/19732, hereby incorporated by reference, is also contemplated. Because these molecules have no free ends, they are more resistant to degradation by exonucleases than are conventional oligonucleotides. These oligonucleotides may be multifunctional, interacting with several regions which are not adjacent to the target mRNA.

**[0510]** The appropriate level of antisense nucleic acids required to inhibit gene expression may be determined using in vitro expression analysis. The antisense molecule may be introduced into the cells by diffusion, injection, infection or transfection using procedures known in the art. For example, the antisense nucleic acids can be introduced into the body as a bare or naked oligonucleotide, oligonucleotide encapsulated in lipid, oligonucleotide sequence encapsulated by viral protein, or as an oligonucleotide operably linked to a promoter contained in an expression vector. The expression vector may be any of a variety of expression vectors known in the art, including retroviral or viral vectors, vectors capable of extrachromosomal replication, or integrating vectors. The vectors may be DNA or RNA.

**[0511]** The antisense molecules are introduced onto cell samples at a number of different concentrations preferably between $1x10^{-10}$M to $1x10^{-4}$M. Once the minimum concentration that can adequately control gene expression is identified, the optimized dose is translated into a dosage suitable for use in vivo. For example, an inhibiting concentration in culture of $1x10^{-7}$ translates into a dose of approximately 0.6 mg/kg bodyweight. Levels of oligonucleotide approaching 100 mg/kg bodyweight or higher may be possible after testing the toxicity of the oligonucleotide in laboratory animals. It is additionally contemplated that cells from the vertebrate are removed, treated with the antisense oligonucleotide, and reintroduced into the vertebrate.

**[0512]** It is further contemplated that the antisense oligonucleotide sequence is incorporated into a ribozyme sequence to enable the antisense to specifically bind and cleave its target mRNA. For technical applications of ribozyme and antisense oligonucleotides see Rossi et al., *supra*.

**[0513]** In a preferred application of this invention, the polypeptide encoded by the gene is first identified, so that the effectiveness of antisense inhibition on translation

can be monitored using techniques that include but are not limited to antibody-mediated tests such as RIAs and ELISA, functional assays, or radiolabeling.

**[0514]** The extended cDNAs of the present invention (or genomic DNAs obtainable therefrom) may also be used in gene therapy approaches based on intracellular triple helix formation. Triple helix oligonucleotides are used to inhibit transcription from a genome. They are particularly useful for studying alterations in cell activity as it is associated with a particular gene. The extended cDNAs (or genomic DNAs obtainable therefrom) of the present invention or, more preferably, a portion of those sequences, can be used to inhibit gene expression in individuals having diseases associated with expression of a particular gene. Similarly, a portion of the extended cDNA (or genomic DNA obtainable therefrom) can be used to study the effect of inhibiting transcription of a particular gene within a cell. Traditionally, homopurine sequences were considered the most useful for triple helix strategies. However, homopyrimidine sequences can also inhibit gene expression. Such homopyrimidine oligonucleotides bind to the major groove at homopurine:homopyrimidine sequences. Thus, both types of sequences from the extended cDNA or from the gene corresponding to the extended cDNA are contemplated within the scope of this invention.

## EXAMPLE 60

Preparation and use of Triple Helix Probes

**[0515]** The sequences of the extended cDNAs (or genomic DNAs obtainable therefrom) are scanned to identify 10-mer to 20-mer homopyrimidine or homopurine stretches which could be used in triple-helix based strategies for inhibiting gene expression. Following identification of candidate homopyrimidine or homopurine stretches, their efficiency in inhibiting gene expression is assessed by introducing varying amounts of oligonucleotides containing the candidate sequences into tissue culture cells which normally express the target gene. The oligonucleotides may be prepared on an oligonucleotide synthesizer or they may be purchased commercially from a company specializing in custom oligonucleotide synthesis, such as GENSET, Paris, France.

**[0516]** The oligonucleotides may be introduced into the cells using a variety of methods known to those skilled in the art, including but not limited to calcium phosphate precipitation, DEAE-Dextran, electroporation, liposome-mediated transfection or native uptake.

**[0517]** Treated cells are monitored for altered cell function or reduced gene expression using techniques such as Northern blotting, RNase protection assays, or PCR based strategies to monitor the transcription levels of the target gene in cells which have been treated with the oligonucleotide . The cell functions to be monitored are predicted based upon the homologies of the target gene corresponding to the extended cDNA from which the oligonucleotide was derived with known gene sequences that have been associated with a particular function. The cell functions can also be predicted based on the presence of abnormal physiologies within cells derived from individuals with a particular inherited disease, particularly when the extended cDNA is associated with the disease using techniques described in Example 53.

**[0518]** The oligonucleotides which are effective in inhibiting gene expression in tissue culture cells may then be introduced in vivo using the techniques described above and in Example 59 at a dosage calculated based on the in vitro results, as described in Example 59.

**[0519]** In some embodiments, the natural (beta) anomers of the oligonucleotide units can be replaced with alpha anomers to render the oligonucleotide more resistant to nucleases. Further, an intercalating agent such as ethidium bromide, or the like, can be attached to the 3′ end of the alpha oligonucleotide to stabilize the triple helix. For information on the generation of oligonucleotides suitable for triple helix formation see Griffin et al. (**Science 245**:967-971 (1989), which is hereby incorporated by this reference).

## EXAMPLE 61

Use of Extended cDNAs to Express an Encoded Protein in a Host Organism

**[0520]** The extended cDNAs of the present invention may also be used to express an encoded protein in a host organism to produce a beneficial effect. In such procedures, the encoded protein may be transiently expressed in the host organism or stably expressed in the host organism. The encoded protein may have any of the activities described above. The encoded protein may be a protein which the host organism lacks or, alternatively, the encoded protein may augment the existing levels of the protein in the host organism.

**[0521]** A full length extended cDNA encoding the signal peptide and the mature protein, or an extended cDNA encoding only the mature protein is introduced into the host organism. The extended cDNA may be introduced into the host organism using a variety of techniques known to those of skill in the art. For example, the extended cDNA may be injected into the host organism as naked DNA such that the encoded protein is expressed in the host organism, thereby producing a beneficial effect.

**[0522]** Alternatively, the extended cDNA may be cloned into an expression vector downstream of a promoter which is active in the host organism. The expression vector may be any of the expression vectors designed for use in gene therapy, including viral or retroviral vectors.

**[0523]** The expression vector may be directly introduced into the host organism such that the encoded protein is expressed in the host organism to produce a beneficial effect. In another approach, the expression vector may be introduced into cells

in vitro. Cells containing the expression vector are thereafter selected and introduced into the host organism, where they express the encoded protein to produce a beneficial effect.

## EXAMPLE 62

Use Of Signal Peptides Encoded By 5' Ests Or Sequences Obtained Therefrom To Import Proteins Into Cells

**[0524]** The short core hydrophobic region (h) of signal peptides encoded by the 5'ESTS or extended cDNAs derived from the 5'ESTs of the present invention may also be used as a carrier to import a peptide or a protein of interest, so-called cargo, into tissue culture cells (Lin *et al., J. Biol. Chem.*, **270**: 14225-14258 (1995); Du *et al., J. Peptide Res.*, **51**: 235-243 (1998); Rojas *et al., Nature Biotech.*, **16**: 370-375 (1998)).

**[0525]** When cell permeable peptides of limited size (approximately up to 25 amino acids) are to be translocated across cell membrane, chemical synthesis may be used in order to add the h region to either the C-terminus or the N-terminus to the cargo peptide of interest. Alternatively, when longer peptides or proteins are to be imported into cells, nucleic acids can be genetically engineered, using techniques familiar to those skilled in the art, in order to link the extended cDNA sequence encoding the h region to the 5' or the 3' end of a DNA sequence coding for a cargo polypeptide. Such genetically engineered nucleic acids are then translated either *in vitro* or *in vivo* after transfection into appropriate cells, using conventional techniques to produce the resulting cell permeable polypeptide. Suitable hosts cells are then simply incubated with the cell permeable polypeptide which is then translocated across the membrane.

**[0526]** This method may be applied to study diverse intracellular functions and cellular processes. For instance, it has been used to probe functionally relevant domains of intracellular proteins and to examine protein-protein interactions involved in signal transduction pathways (Lin *et al., supra*; Lin *et al., J. Biol. Chem.*, **271**: 5305-5308 (1996); Rojas *et al., J. Biol. Chem.*, **271**: 27456-27461 (1996); Liu *et al., Proc. Natl. Acad. Sci. USA*, **93**: 11819-11824 (1996); Rojas *et al., Bioch. Biophys. Res. Commun.*, **234**: 675-680 (1997)).

**[0527]** Such techniques may be used in cellular therapy to import proteins producing therapeutic effects. For instance, cells isolated from a patient may be treated with imported therapeutic proteins and then re-introduced into the host organism.

**[0528]** Alternatively, the h region of signal peptides of the present invention could be used in combination with a nuclear localization signal to deliver nucleic acids into cell nucleus. Such oligonucleotides may be antisense oligonucleotides or oligonucleotides designed to form triple helixes, as described in examples 59 and 60 respectively, in order to inhibit processing and maturation of a target cellular RNA.

## EXAMPLE 63

Reassembling & Resequencing of Clones

**[0529]** Full length cDNA clones obtained by the procedure described in Example 27 were double-sequenced. These sequences were assembled and the resulting consensus sequences were then reanalyzed. Open reading frames were reassigned following essentially the same process as the one described in Example 27.

**[0530]** After this reanalysis process a few abnormalities were revealed. The sequence presented in SEQ ID NO: 84 is apparently unlikely to be genuine full length cDNAs. This clone is more probably a 3' truncated cDNA sequence based on homology studies with existing protein sequences. Similarly, the sequences presented in SEQ ID NOs: 60, 76, 83 and 84 may also not be genuine full length cDNAs based on homology studies with existing protein sequences. Although these sequences encode a potential start methionine, except for SEQ ID NO:60, they could represent a 5' truncated cDNA.

**[0531]** Finally, after the reassignment of open reading frames for the clones, new open reading frames were chosen in some instances. For example, in the case of SEQ ID NOs: 60, 74 and 83 the new open reading frames were no longer predicted to contain a signal peptide.

**[0532]** As discussed above, Table IV provides the sequence identification numbers of the extended cDNAs of the present invention, the locations of

the full coding sequences in SEQ ID NOs: 40-84 and 130-154 (i.e. the nucleotides encoding both the signal peptide and the mature protein, listed under the heading FCS location in Table IV), the locations of the nucleotides in SEQ ID NOs: 40-84 and 130-154 which encode the signal peptides (listed under the heading SigPep Location in Table IV), the locations of the nucleotides in SEQ ID NOs: 40-84 and 130-154 which encode the mature proteins generated by cleavage of the signal peptides (listed under the heading Mature Polypeptide Location in Table IV), the locations in SEQ ID NOs: 40-84 and 130-154 of stop codons (listed under the heading Stop Codon Location in Table IV) the locations in SEQ ID NOs: 40-84 and 130-154 of polyA signals (listed under the heading g PolyA Signal Location in Table IV) and the locations of polyA sites (listed under the heading PolyA Site Location in Table IV).

**[0533]** As discussed above, Table V lists the sequence identification numbers of the polypeptides of SEQ ID NOs: 85-129 and 155-179, the locations of the amino acid residues of SEQ ID NOs: 85-129 and 155-179 in the full length polypeptide (second column), the locations of the amino acid residues of SEQ ID NOs: 85-129 and 155-179 in the signal peptides (third column), and the locations of the amino acid residues of SEQ ID NOs: 85-129 and 155-179 in the mature polypeptide created by cleaving the signal peptide from the full length polypeptide (fourth column). In Table V, and in the appended sequence listing, the first amino acid of the mature protein resulting from cleavage of the signal peptide is designated as amino acid number 1 and the first amino acid of the signal peptide is designated with the appropriate negative number, in accordance with the regulations governing sequence listings.



## Example 64

Functional Analysis of Predicted Protein Sequences

**[0534]** Following double-sequencing, new contigs were assembled for each of the extended cDNAs of the present invention and each was compared to known sequences available at the time of filing. These sequences originate from the following databases : Genbank (release 108 and daily releases up to October, 15, 1998), Genseq (release 32) PIR (release 53) and Swissprot (release 35). The predicted proteins of the

present invention matching known proteins were further classified into 3 categories depending on the level of homology.

**[0535]** The first category contains proteins of the present invention exhibiting more than 80% identical amino acid residues on the whole length of the matched protein. They are clearly close homologues which most probably have the same function or a very similar function as the matched protein.

**[0536]** The second category contains proteins of the present invention exhibiting more remote homologies (30 to 80% over the whole protein) indicating that the protein of the present invention is susceptible to have a function similar to the one of the matched protein.

**[0537]** The third category contains proteins exhibiting either high homology (90 to 100%) to a short domain or more remote homology (40 to 60%) to a larger domain of a known protein indicating that the matched protein and the protein of the invention may share similar features.

**[0538]** It should be noted that the numbering of amino acids in the protein sequences discussed in Figures 10 to 12, and Table VIII, the first methionine encountered is designated as amino acid number 1. In the appended sequence listing, the first amino acid of the mature protein resulting from cleavage of the signal peptide is designated as amino acid number 1 and the first amino acid of the signal peptide is designated with the appropriate negative number, in accordance with the regulations governing sequence listings.

**[0539]** In addition, all of the corrected amino acid sequences (SEQ ID NOs: 85-129 and 155-179) were scanned for the presence of known protein signatures and motifs. This search was performed against the Prosite 15.0 database, using the Proscan software from the GCG package. Functional signatures and their locations are indicated in Table VIII.

**A) Proteins which are closely related to known proteins**

Protein of SEQ ID NO: 120 (internal designation 26-44-1-B5-CL3_1)

**[0540]** The protein of SEQ ID NO: 120 encoded by the extended cDNA SEQ ID NO: 75 isolated from ovary shows extensive homology to a human protein called phospholemman or PLM and its homologues in rodent and canine species. PLM is encoded by the nucleic acid sequence of Genbank accession number U72245 and has the amino acid sequence of SEQ ID NO : 180. Phospholemman is a prominent plasma membrane protein whose phosphorylation correlates with an increase in contractility of myocardium and skeletal muscle. Initially described as a simple chloride channel, it has recently been shown to be a channel for taurine that acts as an osmolyte in the regulation of cell volume (Moorman *et al, Adv Exp. Med. Biol.*, **442**:219-228 (1998)).

**[0541]** As shown by the alignment in Figure 10 between the protein of SEQ ID NO:120 and PLM, the amino acid residues are identical except for positions 3 and 5 in the 92 amino acid long matched protein. The substitution of a proline residue at position 3 par another neutral residue, serine, is conservative. In addition, the protein of the invention also exhibits the typical ATP1G /PLM/MAT8 PROSITE signature (position 27 to 40 in bold in Figure 10) for a family containing mostly proteins known to be either chloride channels or chloride channel regulators In addition, the protein of invention contains 2 short transmembrane segments from positions 1 to 21 and from 37 to 57 as predicted by the software TopPred II (Claros and von Heijne, *CABIOS applic. Notes*, **10** :685-686 (1994)). The first segment (in italic) corresponds to the signal peptide of PLM and the second transmembrane domains (underlined) matches the transmembrane region (double-underlined) shown to be the chloride channel itself (Chen *et al., Circ. Res.*, **82**:367-374 (1998)).

**[0542]** Taken together, these data suggest that the protein of SEQ ID NO 120 may be involved in the regulation of cell volume and in tissue contractility. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, diarrhea, fertility disorders, and in contractility disorders including muscle disorders, pulmonary disorders and myocardial disorders.

Proteins of SEQ ID NOs: 121 (internal designation 47-4-4-C6-CL2_3)

**[0543]** The protein of SEQ ID NO: 121 encoded by the extended cDNA SEQ ID NO: 76 found in substantia nigra shows extensive homology with the human E25 protein. The E25 protein is encoded by the nucleic acid sequence of Genbank accession number AF038953 and has the amino acid sequence of SEQ ID NO: 181. The matched protein might be involved in the development and differentiation of haematopoietic stem/progenitor cells. In addition, it is the human homologue of a murine protein thought to be involved in chondro-osteogenic differentiation and belonging to a novel multigene family of integral membrane proteins (Deleersnijder *et al, J. Biol. Chem.*, 271 :19475-19482 (1996)).

**[0544]** As shown by the alignments in Figure 11 between the protein of SEQ ID NO:121 and E25, the amino acid residues are identical except for positions 9, 24 and 121 in the 263 amino acid long matched sequence. All these substitutions are conservative. In addition, the protein of invention contains one short transmembrane segment from positions 1 to 21 (underlined in Figure 11) matching the one predicted for the murine E25 protein as predicted by the software TopPred II (Claros and von Heijne, *CABIOS applic. Notes*, **10** :685-686 (1994)).

**[0545]** Taken together, these data suggest that the protein of SEQ ID NO: 121 may be involved in cellular proliferation and differentiation, and/or in haematopoiesis. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, hematological, chondro-osteogenic and embryogenetic disorders.

Proteins of SEQ ID NO: 128 (internal designation 58-34-2-H8-CL1_3)

**[0546]** The protein of SEQ ID NO: 128 encoded by the extended cDNA SEQ ID NO: 83 isolated from kidney shows extensive homology to the murine WW-domain binding protein 1 or WWBP-1. WWBP-1 is encoded by the nucleic acid sequence of Genbank accession number U40825 and has the amino acid sequence of SEQ ID NO : 182. This protein is expressed in placenta, lung, liver and kidney is thought to play a role in intracellular signaling by binding to the WW domain of the Yes protooncogene-associated protein via its so-called PY domain (Chen and Sudol, *Proc.*

*Natl. Acad. Sci.*, **92** :7819-7823 (1995)). The WW – PY domains are thought to represent a new set of modular protein-binding sequences just like the SH3 – PXXP domains (Sudol *et al., FEBS Lett.*, **369** :67-71 (1995)).

**[0547]** As shown by the alignments of Figure 12 between the protein of SEQ ID NO:128 and WWBP-1, the amino acid residues are identical to those of the 305 amino acid long matched protein except for positions 53, 66, 78, 89, 92, 94, 96, 100, 102, 106, 110, 113, 124, 128, 136, 139, 140, 142-144, 166, 168, 173, 176, 178, 181, 182, 188, 196, 199, 201, 202, 207 and 210 of the matched protein. 68% of these substitutions are conservative. Indeed the histidine-rich PY domain is present in the protein of the invention (positions 82-86 in bold in Figure 12).

**[0548]** Taken together, these data suggest that the protein of SEQ ID NO: 128 may play a role in intracellular signaling. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, neurodegenerative diseases, cardiovascular disorders, hypertension, renal injury and repair and septic shock.

**B) Proteins which are remotely related to proteins with known functions**

Protein of SEQ ID NO: 97 (internal designation 108-004-5-0-G6-FL)

**[0549]** The protein SEQ ID NO: 97 found in liver encoded by the extended cDNA SEQ ID NO: 52 shows homology to a lectin-like oxidized LDL receptor (LOX-1) found in human, bovine and murine species. Such type II proteins with a C-lectin-like domain, expressed in vascular endothelium and vascular-rich organs, bind and internalize oxidatively modified low-density lipoproteins (Sawamura *et al*, *Nature*, **386**:73-77, (1997)). The oxidized lipoproteins have been implicated in the pathogenesis of atherosclerosis, a leading cause of death in industrialized countries (see review by Parthasarathy *et al, Biochem. Pharmacol.* **56**:279-284 (1998)). In addition, type II membrane proteins with a C-terminus C-type lectin domain, also known as carbohydrate-recognition domains, also include proteins involved in target-cell recognition and cell activation.

**[0550]** The protein of invention has the typical structure of a type II protein belonging to the C-type lectin family. Indeed, it contains a short 31-amino-acid-

long N-terminal tail, a transmembrane segment from positions 32 to 52 matching the one predicted for human LOX-1 and a large 177-amino-acid-long C-terminal tail as predicted by the software TopPred II (Claros and von Heijne, *CABIOS applic. Notes*, **10** :685-686 (1994)). All six cysteines of LOX-1 C-type lectin domain are also conserved in the protein of the invention (positions 102, 113, 130, 195, 208 and 216) although the characteristic PROSITE signature of this family is not. The LOX-1 protein is encoded by the nucleic acid sequence of Genbank accession number: AB010710.

**[0551]** Taken together, these data suggest that the protein of SEQ ID NO: 97 may be involved in the metabolism of lipids and/or in cell-cell or cell-matrix interactions and/or in cell activation. Thus, this protein or part therein, may be useful in diagnosing and treating several disorders including, but not limited to, cancer, hyperlipidaemia, cardiovascular disorders and neurodegenerative disorders.

Protein of SEQ ID NO: 111 (internal designation 108-008-5-0-G12-FL)

**[0552]** The protein SEQ ID NO: 111 encoded by the extended cDNA SEQ ID NO:66 shows homology to a mitochondrial protein found in *Saccharomyces Cerevisiae* (PIR:S72254) which is similar to *E. Coli* ribosomal protein L36. The typical PROSITE signature for ribosomal L36 is present in the protein of the invention (positions 76-102) except for a substitution of a tryptophane residue instead of a valine, leucine, isoleucine, methionine or asparagine residue.

**[0553]** Taken together, these data suggest that the protein of SEQ ID NO: 111 may be involved in protein biosynthesis. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer.

Protein of SEQ ID NO: 94 (internal designation 108-004-5-0-D10-FL)

**[0554]** The protein SEQ ID NO: 94 encoded by the extended cDNA SEQ ID NO: 49 shows remote homology to a subfamily of beta4-galactosyltransferases widely conserved in animals (human, rodents, cow and chicken). Such enzymes, usually type II membrane proteins located in the endoplasmic reticulum or in the Golgi apparatus, catalyzes the biosynthesis of glycoproteins, glycolipid glycans and lactose. Their characteristic features defined as those of subfamily A in Breton *et al, J.*

*Biochem.*, **123**:1000-1009 (1998) are pretty well conserved in the protein of the invention, especially the region I containing the DVD motif (positions 163-165) thought to be involved either in UDP binding or in the catalytic process itself.

**[0555]** In addition, the protein of invention has the typical structure of a type II protein. Indeed, it contains a short 28-amino-acid-long N-terminal tail, a transmembrane segment from positions 29 to 49 and a large 278-amino-acid-long C-terminal tail as predicted by the software TopPred II (Claros and von Heijne, *CABIOS applic. Notes*, **10** :685-686 (1994)).

**[0556]** Taken together, these data suggest that the protein of SEQ ID NO: 94 may play a role in the biosynthesis of polysaccharides, and of the carbohydrate moieties of glycoproteins and glycolipids and/or in cell-cell recognition. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, atherosclerosis, cardiovascular disorders, autoimmune disorders and rheumatic diseases including rheumatoid arthritis.

Protein of SEQ ID NO: 104 (internal designation 108-006-5-0-G2-FL)

**[0557]** The protein of SEQ ID NO: 104 encoded by the extended cDNA SEQ ID NO: 59 shows homology to a neuronal murine protein NP15.6 whose expression is developmentally regulated. NP15.6 protein is encoded by the nucleic acid sequence of Genbank accession number Y08702.

**[0558]** Taken together, these data suggest that the protein of SEQ ID NO: 104 may be involved in cellular proliferation and differentiation. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, neurodegenerative disorders and embryogenetic disorders.

**C) Proteins homologous to a domain of a protein with known function**

Protein of SEQ ID NO: 113 (internal designation 108-009-5-0-A2-FL)

**[0559]** The protein of SEQ ID NO: 113 encoded by the extended cDNA SEQ ID NO: 68 shows extensive homology to the bZIP family of transcription factors, and especially to the human luman protein. (Lu *et al., Mol. Cell. Biol.*, **17** :5117-5126 (1997)). The human luman protein is encoded by the nucleic acid sequence of Genbank

accession number : AF009368. The match include the whole bZIP domain composed of a basic DNA-binding domain and of a leucine zipper allowing protein dimerization. The basic domain is conserved in the protein of the invention as shown by the characteristic PROSITE signature (positions 224-237) except for a conservative substitution of a glutamic acid with an aspartic acid in position 233. The typical PROSITE signature for leucine zipper is also present (positions 259 to 280). Secreted proteins may have nucleic acid binding domain as shown by a nematode protein thought to regulate gene expression which exhibits zinc fingers as well as a functional signal peptide (Holst and Zipfel, *J. Biol. Chem.*, 271 :16275-16733, 1996).

**[0560]** Taken together, these data suggest that the protein of SEQ ID NO: 113 may bind to DNA, hence regulating gene expression as a transcription factor. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer.

Proteins of SEQ ID NO: 129 (internal designation 76-13-3-A9-CL1_1)

**[0561]** The protein of SEQ ID NO: 129 encoded by the extended cDNA SEQ ID NO: 84 shows homology with part of a human seven transmembrane protein. The human seven transmembrane protein is encoded by the nucleic acid sequence of Genbank accession number Y11395. The matched protein potentially associated to stomatin may act as a G-protein coupled receptor and is likely to be important for the signal transduction in neurons and haematopoietic cells (Mayer *et al, Biochem. Biophys. Acta.*, **1395** :301-308 (1998)).

**[0562]** Taken together, these data suggest that the protein of SEQ ID NO: 129 may be involved in signal transduction. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, neurodegenerative diseases, cardiovascular disorders, hypertension, renal injury and repair and septic shock.

Proteins of SEQ ID NO: 95 (internal designation 108-004-5-0-E8-FL)

**[0563]** The protein of SEQ ID NO: 95 encoded by the extended cDNA SEQ ID NO: 50 exhibit the typical PROSITE signature for amino acid permeases (positions 5 to 66) which are integral membrane proteins involved in the transport of

amino acids into the cell. In addition, the protein of invention has a transmembrane segment from positions 9 to 29 as predicted by the software TopPred II (Claros and von Heijne, *CABIOS applic. Notes*, **10** :685-686 (1994)).

**[0564]** Taken together, these data suggest that the protein of SEQ ID NO: 95 may be involved in amino acid transport. Thus, this protein may be useful in diagnosing and/or treating several types of disorders including, but not limited to, cancer, aminoacidurias, neurodegenerative diseases, anorexia, chronic fatigue, coronary vascular disease, diphtheria, hypoglycemia, male infertility, muscular and myopathies.

**[0565]** As discussed above, the extended cDNAs of the present invention or portions thereof can be used for various purposes. The polynucleotides can be used to express recombinant protein for analysis, characterization or therapeutic use; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in disease states); as molecular weight markers on Southern gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; for selecting and making oligomers for attachment to a "gene chip" or other support, including for examination for expression patterns; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or elicit another immune response. Where the polynucleotide encodes a protein which binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the polynucleotide can also be used in interaction trap assays (such as, for example, that described in Gyuris et al., Cell 75:791-803 (1993)) to identify polynucleotides encoding the other protein with which binding occurs or to identify inhibitors of the binding interaction.

**[0566]** The proteins or polypeptides provided by the present invention can similarly be used in assays to determine biological activity, including in a panel of multiple proteins for high-throughput screening; to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its receptor) in biological fluids; as markers for tissues

in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state); and, of course, to isolate correlative receptors or ligands. Where the protein binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the protein can be used to identify the other protein with which binding occurs or to identify inhibitors of the binding interaction. Proteins involved in these binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction.

**[0567]** Any or all of these research utilities are capable of being developed into reagent grade or kit format for commercialization as research products.

**[0568]** Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include without limitation "Molecular Cloning; A Laboratory Manual", 2d ed., Cole Spring Harbor Laboratory Press, Sambrook, J., E.F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology; Guide to Molecular Cloning Techniques", Academic Press, Berger, S.L. and A.R. Kimmel eds., 1987.

**[0569]** Polynucleotides and proteins of the present invention can also be used as nutritional sources or supplements. Such uses include without limitation use as a protein or amino acid supplement, use as a carbon source, use as a nitrogen source and use as a source of carbohydrate. In such cases the protein or polynucleotide of the invention can be added to the feed of a particular organism or can be administered as a separate solid or liquid preparation, such as in the form of powder, pills, solutions, suspensions or capsules. In the case of microorganisms, the protein or polynucleotide of the invention can be added to the medium in or on which the microorganism is cultured.

**[0570]** Although this invention has been described in terms of certain preferred embodiments, other embodiments which will be apparent to those of ordinary skill in the art in view of the disclosure herein are also within the scope of this invention. Accordingly, the scope of the invention is intended to be defined only by reference to the appended claims. All documents cited herein are incorporated herein by reference in their entirety.

**TABLE I**

| SEQ ID NO. in Present Application | Provisional Application Disclosing Sequence | SEQ ID NO. in Provisional Application |
|---|---|---|
| 40 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 40 |
| 41 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 41 |
| 42 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 62 |
| 43 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 47 |
| 44 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 43 |
| 45 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 42 |
| 46 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 43 |
| 47 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 45 |
| 48 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 44 |
| 49 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 50 |
| 50 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 49 |
| 51 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 44 |
| 52 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 45 |
| 53 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 46 |
| 54 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 51 |
| 55 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 59 |
| 56 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 61 |
| 57 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 53 |
| 58 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 52 |
| 59 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 54 |
| 60 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 47 |
| 61 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 63 |
| 62 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 46 |
| 63 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 48 |
| 64 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 58 |
| 65 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 56 |
| 66 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 49 |
| 67 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 57 |
| 68 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 55 |
| 69 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 42 |
| 70 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 41 |
| 71 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 48 |
| 72 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 60 |
| 73 | U.S. Application No. 60/096,116, filed on August 10, 1998 | 50 |
| 74 | U.S. Application No. 60/099,273, filed on September 4, 1998 | 40 |
| 75 | U.S. Application No. 60/074,121, filed on February 9, 1998 | 42 |
| 76 | U.S. Application No. 60/074,121, filed on February 9, 1998 | 56 |
| 77 | U.S. Application No. 60/074,121, filed on February 9, 1998 | 57 |

| SEQ ID NO. in Present Application | Provisional Application Disclosing Sequence | SEQ ID NO. in Provisional Application |
|---|---|---|
| 78 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 84 |
| 79 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 69 |
| 80 | U.S. Application No. 60/074,121, filed on February 9, 1998 | 62 |
| 81 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 79 |
| 82 | U.S. Application No. 60/074,121, filed on February 9, 1998 | 64 |
| 83 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 51 |
| 84 | U.S. Application No. 60/074,121, filed on February 9, 1998 | 71 |
| 130 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 40 |
| 131 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 41 |
| 132 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 42 |
| 133 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 43 |
| 134 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 44 |
| 135 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 45 |
| 136 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 46 |
| 137 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 47 |
| 138 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 48 |
| 139 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 49 |
| 140 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 50 |
| 141 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 53 |
| 142 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 54 |
| 143 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 55 |
| 144 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 56 |
| 145 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 57 |
| 146 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 58 |
| 147 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 59 |
| 148 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 60 |
| 149 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 61 |
| 150 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 62 |
| 151 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 63 |
| 152 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 64 |
| 153 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 65 |
| 154 | U.S. Application No. 60/081,563, filed on April 13, 1998 | 66 |

**TABLE II**

Parameters used for each step of EST analysis

| | Search Characteristics | | | Selection Characteristics | |
|---|---|---|---|---|---|
| Step | Program | Strand | Parameters | Identity (%)) | Length (bp) |
| Miscellaneous | Blastn | both | S=61 X=16 | 90 | 17 |
| tRNA | Fasta | both | - | 80 | 60 |
| rRNA | Blastn | both | S=108 | 80 | 40 |
| mtRNA | Blastn | both | S=108 | 80 | 40 |
| Procaryotic | Blastn | both | S=144 | 90 | 40 |
| Fungal | Blastn | both | S=144 | 90 | 40 |
| Alu | fasta* | both | - | 70 | 40 |
| L1 | Blastn | both | S=72 | 70 | 40 |
| Repeats | Blastn | both | S=72 | 70 | 40 |
| Promoters | Blastn | top | S=54 X=16 | 90 | 15⊥ |
| Vertebrate | fasta* | both | S=108 | 90 | 30 |
| ESTs | Blatsn | both | S=108 X=16 | 90 | 30 |
| Proteins | blastxη | top | E=0.001 | - | - |

* use "Quick Fast" Database Scanner

⊥ alignment further constrained to begin closer than 10bp to EST\5' end

η using BLOSUM62 substitution matrix

## TABLE III

Parameters used for each step of extended cDNA analysis

| | Search characteristics | | Selection characteristics | | | |
|---|---|---|---|---|---|---|
| Step | Program | Strand | Parameters | Identity (%) | Length (bp) | Comments |
| miscellaneous* | FASTA | both | - | 90 | 15 | |
| tRNA[$] | FASTA | both | - | 80 | 90 | |
| rRNA[$] | BLASTN | both | S=108 | 80 | 40 | |
| mtRNA[$] | BLASTN | both | S=108 | 80 | 40 | |
| Procaryotic[$] | BLASTN | both | S=144 | 90 | 40 | |
| Fungal* | BLASTN | both | S=144 | 90 | 40 | |
| Alu* | BLASTN | both | S=72 | 70 | 40 | max 5 matches, masking |
| L1[$] | BLASTN | both | S=72 | 70 | 40 | max 5 matches, masking |
| Repeats[$] | BLASTN | both | S=72 | 70 | 40 | masking |
| PolyA | BLAST2N | top | W=6,S=10,E=1000 | 90 | 8 | in the last 20 nucleotides |
| Polyadenylation signal | - | top | AATAAA allowing 1 mismatch | | | in the 50 nucleotides preceding the 5' end of the polA |
| Vertibrate* | BLASTN then FASTA | both | - | 90 then 70 | 30 | first BLASTN and then FASTA on matching sequences |
| ESTs* | BLAST2N | both | - | 90 | 30 | |
| Geneseq | BLASTN | both | W=8, B=10 | 90 | 30 | |
| ORF | BLASTP | top | W=8, B=10 | - | - | on ORF proteins, max 10 matches |
| Proteins* | BLASTX | top | E=0.001 | 70 | 30 | |

[$] steps common to EST analysis and using the same algorithms and parameters

* steps also used in EST analysis but with different algorithms and/or parameters

**TABLE IV**

| Id | FCS Location | SigPep Location | Mature Polypeptide Location | Stop Codon Location | PolyA Signal Location | PolyA Site Location |
|---|---|---|---|---|---|---|
| 40 | 35 through 568 | 35 through 100 | 101 through 568 | 569 | 667 through 672 | 685 through 699 |
| 41 | 68 through 337 | 68 through 124 | 125 through 337 | 338 | 462 through 467 | 482 through 497 |
| 42 | 39 through 413 | 39 through 83 | 84 through 413 | 414 | 566 through 571 | 583 through 598 |
| 43 | 235 through 642 | 235 through 336 | 337 through 642 | 643 | 1540 through 1545 | 1564 through 1579 |
| 44 | 42 through 755 | 42 through 200 | 201 through 755 | 756 | 860 through 865 | 878 through 893 |
| 45 | 23 through 340 | 23 through 235 | 236 through 340 | 341 | 611 through 616 | 629 through 644 |
| 46 | 12 through 380 | 12 through 263 | 264 through 380 | 381 | - | 523 through 538 |
| 47 | 8 through 232 | 8 through 154 | 155 through 232 | 233 | - | 737 through 752 |
| 48 | 183 through 422 | 183 through 302 | 303 through 422 | 423 | 505 through 510 | 523 through 537 |
| 49 | 24 through 1004 | 24 through 170 | 171 through 1004 | 1005 | - | 1586 through 1602 |
| 50 | 80 through 784 | 80 through 139 | 140 through 784 | 785 | 910 through 915 | 933 through 948 |
| 51 | 67 through 222 | 67 through 159 | 160 through 222 | 223 | - | 673 through 687 |
| 52 | 46 through 732 | 46 through 186 | 187 through 732 | 733 | 781 through 786 | 806 through 821 |
| 53 | 81 through 356 | 81 through 152 | 153 through 356 | 357 | 406 through 411 | 429 through 445 |
| 54 | 72 through 1346 | 72 through 140 | 141 through 1346 | 1347 | 1482 through 1487 | 1502 through 1517 |
| 55 | 194 through 454 | 194 through 379 | 380 through 454 | 455 | - | 1545 through 1560 |
| 56 | 48 through 494 | 48 through 347 | 348 through 494 | 495 | 1031 through 1036 | 1051 through 1066 |
| 57 | 111 through 671 | 111 through 215 | 216 through 671 | 672 | 990 through 995 | 1045 through 1061 |
| 58 | 5 through 373 | 5 through 82 | 83 through 373 | 374 | 1986 through 1991 | 2010 through 2025 |
| 59 | 14 through 472 | 14 through 319 | 320 through 472 | 473 | 555 through 560 | 576 through 591 |
| 60 | 2 through 217 | - | 2 through 217 | 218 | 489 through | 529 through |

| Id | FCS Location | SigPep Location | Mature Polypeptide Location | Stop Codon Location | PolyA Signal Location | PolyA Site Location |
|---|---|---|---|---|---|---|
| | | | | | 494 | 544 |
| 61 | 51 through 575 | 51 through 110 | 111 through 575 | 576 | 1653 through 1658 | 1674 through 1689 |
| 62 | 69 through 977 | 69 through 128 | 129 through 977 | 978 | 1076 through 1081 | 1096 through 1111 |
| 63 | 44 through 238 | 44 through 160 | 161 through 238 | 239 | 443 through 448 | 540 through 554 |
| 64 | 114 through 524 | 114 through 164 | 165 through 524 | 525 | 1739 through 1744 | 1758 through 1773 |
| 65 | 26 through 487 | 26 through 64 | 65 through 487 | 488 | 883 through 888 | 901 through 917 |
| 66 | 80 through 388 | 80 through 187 | 188 through 388 | 389 | 609 through 614 | 627 through 641 |
| 67 | 186 through 443 | 186 through 407 | 408 through 443 | 444 | 827 through 832 | 839 through 854 |
| 68 | 75 through 1259 | 75 through 1004 | 1005 through 1259 | 1260 | 1536 through 1541 | 1553 through 1568 |
| 69 | 98 through 376 | 98 through 151 | 152 through 376 | 377 | 471 through 476 | 491 through 506 |
| 70 | 72 through 254 | 72 through 134 | 135 through 254 | 255 | 506 through 511 | 528 through 542 |
| 71 | 148 through 1140 | 148 through 240 | 241 through 1140 | 1141 | 1590 through 1595 | 1614 through 1629 |
| 72 | 109 through 738 | 109 through 405 | 406 through 738 | 739 | 1633 through 1638 | 1650 through 1665 |
| 73 | 55 through 291 | 55 through 255 | 256 through 291 | 292 | 390 through 395 | 410 through 425 |
| 74 | 25 through 276 | - | 25 through 276 | 277 | 508 through 513 | 533 through 546 |
| 75 | 32 through 307 | 32 through 91 | 92 through 307 | 308 | 452 through 457 | 472 through 485 |
| 76 | 46 through 675 | 46 through 87 | 88 through 675 | 676 | 1363 through 1368 | 1382 through 1394 |
| 77 | 329 through 943 | 329 through 745 | 746 through 943 | 944 | - | 1322 through 1333 |
| 78 | 27 through 281 | 27 through 77 | 78 through 281 | 282 | - | - |
| 79 | 61 through 405 | 61 through 213 | 214 through 405 | 406 | 675 through 680 | 692 through 703 |
| 80 | 137 through 379 | 137 through 229 | 230 through 379 | 380 | 728 through 733 | 755 through 768 |
| 81 | 37 through | 37 through 153 | 154 through | 742 | 969 through | 994 through |

| Id | FCS Location | SigPep Location | Mature Polypeptide Location | Stop Codon Location | PolyA Signal Location | PolyA Site Location |
|---|---|---|---|---|---|---|
| | 741 | | 741 | | 974 | 1007 |
| 82 | 80 through 265 | 80 through 142 | 143 through 265 | 266 | 491 through 496 | 517 through 527 |
| 83 | 612 through 644 | - | 612 through 644 | 645 | 829 through 834 | 850 through 861 |
| 84 | 61 through 228 | 61 through 162 | 163 through 228 | 229 | 208 through 213 | - |
| 130 | 15 through 311 | 15 through 110 | 111 through 311 | 312 | 507 through 512 | 531 through 542 |
| 131 | 50 through 529 | 50 through 130 | 131 through 529 | 530 | 877 through 882 | 899 through 909 |
| 132 | 240 through 416 | 240 through 305 | 306 through 416 | 417 | 1117 through 1122 | 1139 through 1149 |
| 133 | 111 through 446 | 111 through 254 | 255 through 446 | 447 | 890 through 895 | 909 through 921 |
| 134 | 123 through 455 | 123 through 290 | 291 through 455 | 456 | 886 through 891 | 904 through 916 |
| 135 | 2 through 433 | 2 through 232 | 233 through 433 | 434 | 488 through 493 | 510 through 520 |
| 136 | 34 through 363 | 34 through 87 | 88 through 363 | 364 | 536 through 541 | 558 through 568 |
| 137 | 50 through 286 | 50 through 157 | 158 through 286 | 287 | 385 through 390 | 405 through 416 |
| 138 | 50 through 637 | 50 through 151 | 152 through 637 | 638 | - | 1277 through 1289 |
| 139 | 72 through 602 | 72 through 125 | 126 through 602 | 603 | - | 704 through 715 |
| 140 | 120 through 434 | 120 through 185 | 186 through 434 | 435 | 899 through 904 | 918 through 931 |
| 141 | 4 through 447 | 4 through 147 | 148 through 447 | 448 | 858 through 863 | 880 through 891 |
| 142 | 28 through 804 | 28 through 96 | 97 through 804 | 805 | - | 806 through 817 |
| 143 | 27 through 359 | 27 through 212 | 213 through 359 | 360 | 988 through 993 | 1009 through 1020 |
| 144 | 25 through 957 | 25 through 93 | 94 through 957 | 958 | 1368 through 1373 | 1388 through 1399 |
| 145 | 47 through 319 | 47 through 226 | 227 through 319 | 320 | - | 656 through 666 |
| 146 | 80 through 940 | 80 through 130 | 131 through 940 | 941 | 1101 through 1106 | 1119 through 1130 |
| 147 | 146 through | 146 through | 293 through | 458 | 442 through | 465 through |

| Id | FCS Location | SigPep Location | Mature Polypeptide Location | Stop Codon Location | PolyA Signal Location | PolyA Site Location |
|---|---|---|---|---|---|---|
| | 457 | 292 | 457 | | 447 | 475 |
| 148 | 100 through 351 | 100 through 207 | 208 through 351 | 352 | - | 940 through 949 |
| 149 | 177 through 569 | 177 through 236 | 237 through 569 | 570 | - | 931 through 939 |
| 150 | 67 through 459 | 67 through 135 | 136 through 459 | 460 | 856 through 861 | 875 through 887 |
| 151 | 65 through 1069 | 65 through 112 | 113 through 1069 | 1070 | 1978 through 1983 | 1999 through 2010 |
| 152 | 70 through 321 | 70 through 234 | 235 through 321 | 322 | 364 through 369 | 375 through 387 |
| 153 | 38 through 877 | 38 through 91 | 92 through 877 | 878 | 947 through 952 | 974 through 983 |
| 154 | 51 through 470 | 51 through 203 | 204 through 470 | 471 | 1585 through 1590 | 1604 through 1614 |

**TABLE V**

| Id | Full Length Polypeptide Location | Signal Peptide Location | Mature Polypeptide Location |
|---|---|---|---|
| 85 | -22 through 156 | -22 through -1 | 1 through 156 |
| 86 | -19 through 71 | -19 through -1 | 1 through 71 |
| 87 | -15 through 110 | -15 through -1 | 1 through 110 |
| 88 | -34 through 102 | -34 through -1 | 1 through 102 |
| 89 | -53 through 185 | -53 through -1 | 1 through 185 |
| 90 | -71 through 35 | -71 through -1 | 1 through 35 |
| 91 | -84 through 39 | -84 through -1 | 1 through 39 |
| 92 | -49 through 26 | -49 through -1 | 1 through 26 |
| 93 | -40 through 40 | -40 through -1 | 1 through 40 |
| 94 | -49 through 278 | -49 through -1 | 1 through 278 |
| 95 | -20 through 215 | -20 through -1 | 1 through 215 |
| 96 | -31 through 21 | -31 through -1 | 1 through 21 |
| 97 | -47 through 182 | -47 through -1 | 1 through 182 |
| 98 | -24 through 68 | -24 through -1 | 1 through 68 |
| 99 | -23 through 402 | -23 through -1 | 1 through 402 |
| 100 | -62 through 25 | -62 through -1 | 1 through 25 |
| 101 | -100 through 49 | -100 through -1 | 1 through 49 |
| 102 | -35 through 152 | -35 through -1 | 1 through 152 |
| 103 | -26 through 97 | -26 through -1 | 1 through 97 |
| 104 | -102 through 51 | -102 through -1 | 1 through 51 |
| 105 | 1 through 72 | - | 1 through 72 |
| 106 | -20 through 155 | -20 through -1 | 1 through 155 |
| 107 | -20 through 283 | -20 through -1 | 1 through 283 |
| 108 | -39 through 26 | -39 through -1 | 1 through 26 |
| 109 | -17 through 120 | -17 through -1 | 1 through 120 |
| 110 | -13 through 141 | -13 through -1 | 1 through 141 |
| 111 | -36 through 67 | -36 through -1 | 1 through 67 |
| 112 | -74 through 12 | -74 through -1 | 1 through 12 |
| 113 | -310 through 85 | -310 through -1 | 1 through 85 |
| 114 | -18 through 75 | -18 through -1 | 1 through 75 |
| 115 | -21 through 40 | -21 through -1 | 1 through 40 |
| 116 | -31 through 300 | -31 through -1 | 1 through 300 |
| 117 | -99 through 111 | -99 through -1 | 1 through 111 |
| 118 | -67 through 12 | -67 through -1 | 1 through 12 |
| 119 | 1 through 84 | - | 1 through 84 |
| 120 | -20 through 72 | -20 through -1 | 1 through 72 |

| Id | Full Length Polypeptide Location | Signal Peptide Location | Mature Polypeptide Location |
|---|---|---|---|
| 121 | -14 through 196 | -14 through -1 | 1 through 196 |
| 122 | -139 through 66 | -139 through -1 | 1 through 66 |
| 123 | -17 through 68 | -17 through -1 | 1 through 68 |
| 124 | -51 through 64 | -51 through -1 | 1 through 64 |
| 125 | -31 through 50 | -31 through -1 | 1 through 50 |
| 126 | -39 through 196 | -39 through -1 | 1 through 196 |
| 127 | -21 through 41 | -21 through -1 | 1 through 41 |
| 128 | 1 through 11 | - | 1 through 11 |
| 129 | -34 through 22 | -34 through -1 | 1 through 22 |
| 155 | -32 through 67 | -32 through -1 | 1 through 67 |
| 156 | -27 through 133 | -27 through -1 | 1 through 133 |
| 157 | -22 through 37 | -22 through -1 | 1 through 37 |
| 158 | -48 through 64 | -48 through -1 | 1 through 64 |
| 159 | -56 through 55 | -56 through -1 | 1 through 55 |
| 160 | -77 through 67 | -77 through -1 | 1 through 67 |
| 161 | -18 through 92 | -18 through -1 | 1 through 92 |
| 162 | -36 through 43 | -36 through -1 | 1 through 43 |
| 163 | -34 through 162 | -34 through -1 | 1 through 162 |
| 164 | -18 through 159 | -18 through -1 | 1 through 159 |
| 165 | -22 through 83 | -22 through -1 | 1 through 83 |
| 166 | -48 through 100 | -48 through -1 | 1 through 100 |
| 167 | -23 through 236 | -23 through -1 | 1 through 236 |
| 168 | -62 through 49 | -62 through -1 | 1 through 49 |
| 169 | -23 through 288 | -23 through -1 | 1 through 288 |
| 170 | -60 through 31 | -60 through -1 | 1 through 31 |
| 171 | -17 through 270 | -17 through -1 | 1 through 270 |
| 172 | -49 through 55 | -49 through -1 | 1 through 55 |
| 173 | -36 through 48 | -36 through -1 | 1 through 48 |
| 174 | -20 through 111 | -20 through -1 | 1 through 111 |
| 175 | -23 through 108 | -23 through -1 | 1 through 108 |
| 176 | -16 through 319 | -16 through -1 | 1 through 319 |
| 177 | -55 through 29 | -55 through -1 | 1 through 29 |
| 178 | -18 through 262 | -18 through -1 | 1 through 262 |
| 179 | -51 through 89 | -51 through -1 | 1 through 89 |

**TABLE VI**

| Id | Collection refs | Deposit Name |
|---|---|---|
| 40 | ATCC# 98921 | SignalTag 121-144 |
| 41 | ATCC# 98921 | SignalTag 121-144 |
| 42 | ATCC# 98919 | SignalTag 145-165 |
| 43 | ATCC# 98919 | SignalTag 145-165 |
| 44 | ATCC# 98919 | SignalTag 145-165 |
| 45 | ATCC# 98921 | SignalTag 121-144 |
| 46 | ATCC# 98921 | SignalTag 121-144 |
| 47 | ATCC# 98919 | SignalTag 145-165 |
| 48 | ATCC# 98919 | SignalTag 145-165 |
| 49 | ATCC# 98919 | SignalTag 145-165 |
| 50 | ATCC# 98919 | SignalTag 145-165 |
| 51 | ATCC# 98921 | SignalTag 121-144 |
| 52 | ATCC# 98921 | SignalTag 121-144 |
| 53 | ATCC# 98921 | SignalTag 121-144 |
| 54 | ATCC# 98919 | SignalTag 145-165 |
| 55 | ATCC# 98919 | SignalTag 145-165 |
| 56 | ATCC# 98919 | SignalTag 145-165 |
| 57 | ATCC# 98919 | SignalTag 145-165 |
| 58 | ATCC# 98919 | SignalTag 145-165 |
| 59 | ATCC# 98919 | SignalTag 145-165 |
| 60 | ATCC# 98921 | SignalTag 121-144 |
| 61 | ATCC# 98919 | SignalTag 145-165 |
| 62 | ATCC# 98919 | SignalTag 145-165 |
| 63 | ATCC# 98921 | SignalTag 121-144 |
| 64 | ATCC# 98919 | SignalTag 145-165 |
| 65 | ATCC# 98919 | SignalTag 145-165 |
| 66 | ATCC# 98921 | SignalTag 121-144 |
| 67 | ATCC# 98919 | SignalTag 145-165 |
| 68 | ATCC# 98919 | SignalTag 145-165 |
| 69 | ATCC# 98919 | SignalTag 145-165 |
| 70 | ATCC# 98919 | SignalTag 145-165 |
| 71 | ECACC# XXXX | Signal Tag 28011 999 |
| 72 | ECACC# XXXX | Signal Tag 28011 999 |
| 73 | ECACC# XXXX | Signal Tag 28011 999 |
| 74 | ECACC# XXXX | Signal Tag 28011 999 |
| 75 | ECACC# XXXX | Signal Tag 28011 999 |
| 76 | ECACC# XXXX | Signal Tag 28011 999 |
| 77 | ECACC# XXXX | Signal Tag 28011 999 |
| 78 | ECACC# XXXX | Signal Tag 28011 999 |
| 79 | ECACC# XXXX | Signal Tag 28011 999 |
| 80 | ECACC# XXXX | Signal Tag 28011 999 |
| 81 | ECACC# XXXX | Signal Tag 28011 999 |
| 82 | ECACC# XXXX | Signal Tag 28011 999 |
| 83 | ECACC# XXXX | Signal Tag 28011 999 |
| 84 | ECACC# XXXX | Signal Tag 28011 999 |

**TABLE VII**

| Internal designation | Id | Type of sequence |
|---|---|---|
| 108-002-5-0-B1-FL | 40 | DNA |
| 108-002-5-0-F3-FL | 41 | DNA |
| 108-002-5-0-F4-FL | 42 | DNA |
| 108-003-5-0-A8-FL | 43 | DNA |
| 108-003-5-0-D2-FL | 44 | DNA |
| 108-003-5-0-E5-FL | 45 | DNA |
| 108-003-5-0-H2-FL | 46 | DNA |
| 108-004-5-0-B7-FL | 47 | DNA |
| 108-004-5-0-C8-FL | 48 | DNA |
| 108-004-5-0-D10-FL | 49 | DNA |
| 108-004-5-0-E8-FL | 50 | DNA |
| 108-004-5-0-F5-FL | 51 | DNA |
| 108-004-5-0-G6-FL | 52 | DNA |
| 108-005-5-0-B11-FL | 53 | DNA |
| 108-005-5-0-C1-FL | 54 | DNA |
| 108-005-5-0-F11-FL | 55 | DNA |
| 108-005-5-0-F6-FL | 56 | DNA |
| 108-006-5-0-C2-FL | 57 | DNA |
| 108-006-5-0-E6-FL | 58 | DNA |
| 108-006-5-0-G2-FL | 59 | DNA |
| 108-006-5-0-G4-FL | 60 | DNA |
| 108-008-5-0-A6-FL | 61 | DNA |
| 108-008-5-0-A8-FL | 62 | DNA |
| 108-008-5-0-C10-FL | 63 | DNA |
| 108-008-5-0-E6-FL | 64 | DNA |
| 108-008-5-0-F6-FL | 65 | DNA |
| 108-008-5-0-G12-FL | 66 | DNA |
| 108-008-5-0-G4-FL | 67 | DNA |
| 108-009-5-0-A2-FL | 68 | DNA |
| 108-013-5-0-C12-FL | 69 | DNA |
| 108-013-5-0-G11-FL | 70 | DNA |
| 108-003-5-0-E4-FL | 71 | DNA |
| 108-005-5-0-D6-FL | 72 | DNA |
| 108-008-5-0-G3-FL | 73 | DNA |
| 108-013-5-0-B5-FL | 74 | DNA |
| 26-44-1-B5-CL3_1 | 75 | DNA |
| 47-4-4-C6-CL2_3 | 76 | DNA |
| 47-40-4-G9-CL1_1 | 77 | DNA |
| 48-25-4-D8-CL1_7 | 78 | DNA |
| 48-28-3-A9-CL0_1 | 79 | DNA |
| 51-25-1-A2-CL3_1 | 80 | DNA |
| 55-10-3-F5-CL0_3 | 81 | DNA |
| 57-19-2-G8-CL1_3 | 82 | DNA |
| 58-34-2-H8-CL1_3 | 83 | DNA |
| 76-13-3-A9-CL1_1 | 84 | DNA |
| 78-7-2-B8-FL1 | 130 | DNA |

| Internal designation | Id | Type of sequence |
|---|---|---|
| 77-8-4-F9-FL1 | 131 | DNA |
| 58-8-1-F2-FL2 | 132 | DNA |
| 77-13-1-A7-FL2 | 133 | DNA |
| 47-2-3-G9-FL1 | 134 | DNA |
| 33-75-4-H7-FL1 | 135 | DNA |
| 51-41-1-F10-FL1 | 136 | DNA |
| 48-51-4-C11-FL1 | 137 | DNA |
| 33-58-3-C8-FL1 | 138 | DNA |
| 76-20-4-C11-FL1 | 139 | DNA |
| 76-28-3-A12-FL1 | 140 | DNA |
| 76-25-4-F11-FL1 | 141 | DNA |
| 58-20-4-G7-FL1 | 142 | DNA |
| 33-54-1-B9-FL1 | 143 | DNA |
| 76-20-3-H1-FL1 | 144 | DNA |
| 47-20-2-G3-FL1 | 145 | DNA |
| 78-25-1-H11-FL1 | 146 | DNA |
| 78-6-2-B10-FL1 | 147 | DNA |
| 58-49-3-G10-FL1 | 148 | DNA |
| 78-21-1-B7-FL1 | 149 | DNA |
| 57-28-4-B12-FL1 | 150 | DNA |
| 33-77-4-E2-FL1 | 151 | DNA |
| 58-19-3-D3-FL2 | 152 | DNA |
| 37-7-4-E7-FL1 | 153 | DNA |
| 60-14-2-H10-FL1 | 154 | DNA |
| 108-002-5-0-B1-FL | 85 | PRT |
| 108-002-5-0-F3-FL | 86 | PRT |
| 108-002-5-0-F4-FL | 87 | PRT |
| 108-003-5-0-A8-FL | 88 | PRT |
| 108-003-5-0-D2-FL | 89 | PRT |
| 108-003-5-0-E5-FL | 90 | PRT |
| 108-003-5-0-H2-FL | 91 | PRT |
| 108-004-5-0-B7-FL | 92 | PRT |
| 108-004-5-0-C8-FL | 93 | PRT |
| 108-004-5-0-D10-FL | 94 | PRT |
| 108-004-5-0-E8-FL | 95 | PRT |
| 108-004-5-0-F5-FL | 96 | PRT |
| 108-004-5-0-G6-FL | 97 | PRT |
| 108-005-5-0-B11-FL | 98 | PRT |
| 108-005-5-0-C1-FL | 99 | PRT |
| 108-005-5-0-F11-FL | 100 | PRT |
| 108-005-5-0-F6-FL | 101 | PRT |
| 108-006-5-0-C2-FL | 102 | PRT |
| 108-006-5-0-E6-FL | 103 | PRT |
| 108-006-5-0-G2-FL | 104 | PRT |
| 108-006-5-0-G4-FL | 105 | PRT |
| 108-008-5-0-A6-FL | 106 | PRT |
| 108-008-5-0-A8-FL | 107 | PRT |
| 108-008-5-0-C10-FL | 108 | PRT |
| 108-008-5-0-E6-FL | 109 | PRT |

| Internal designation | Id | Type of sequence |
|---|---|---|
| 108-008-5-0-F6-FL | 110 | PRT |
| 108-008-5-0-G12-FL | 111 | PRT |
| 108-008-5-0-G4-FL | 112 | PRT |
| 108-009-5-0-A2-FL | 113 | PRT |
| 108-013-5-0-C12-FL | 114 | PRT |
| 108-013-5-0-G11-FL | 115 | PRT |
| 108-003-5-0-E4-FL | 116 | PRT |
| 108-005-5-0-D6-FL | 117 | PRT |
| 108-008-5-0-G3-FL | 118 | PRT |
| 108-013-5-0-B5-FL | 119 | PRT |
| 26-44-1-B5-CL3_1 | 120 | PRT |
| 47-4-4-C6-CL2_3 | 121 | PRT |
| 47-40-4-G9-CL1_1 | 122 | PRT |
| 48-25-4-D8-CL1_7 | 123 | PRT |
| 48-28-3-A9-CL0_1 | 124 | PRT |
| 51-25-1-A2-CL3_1 | 125 | PRT |
| 55-10-3-F5-CL0_3 | 126 | PRT |
| 57-19-2-G8-CL1_3 | 127 | PRT |
| 58-34-2-H8-CL1_3 | 128 | PRT |
| 76-13-3-A9-CL1_1 | 129 | PRT |
| 78-7-2-B8-FL1 | 155 | PRT |
| 77-8-4-F9-FL1 | 156 | PRT |
| 58-8-1-F2-FL2 | 157 | PRT |
| 77-13-1-A7-FL2 | 158 | PRT |
| 47-2-3-G9-FL1 | 159 | PRT |
| 33-75-4-H7-FL1 | 160 | PRT |
| 51-41-1-F10-FL1 | 161 | PRT |
| 48-51-4-C11-FL1 | 162 | PRT |
| 33-58-3-C8-FL1 | 163 | PRT |
| 76-20-4-C11-FL1 | 164 | PRT |
| 76-28-3-A12-FL1 | 165 | PRT |
| 76-25-4-F11-FL1 | 166 | PRT |
| 58-20-4-G7-FL1 | 167 | PRT |
| 33-54-1-B9-FL1 | 168 | PRT |
| 76-20-3-H1-FL1 | 169 | PRT |
| 47-20-2-G3-FL1 | 170 | PRT |
| 78-25-1-H11-FL1 | 171 | PRT |
| 78-6-2-B10-FL1 | 172 | PRT |
| 58-49-3-G10-FL1 | 173 | PRT |
| 78-21-1-B7-FL1 | 174 | PRT |
| 57-28-4-B12-FL1 | 175 | PRT |
| 33-77-4-E2-FL1 | 176 | PRT |
| 58-19-3-D3-FL2 | 177 | PRT |
| 37-7-4-E7-FL1 | 178 | PRT |
| 60-14-2-H10-FL1 | 179 | PRT |

**TABLE VIII**

| Id | Locations | PROSITE signature Name |
|---|---|---|
| 89 | 205-226 | Leucine zipper |
| 95 | 5-66 | Amino acid permease |
| 103 | 46-67 | Leucine zipper |
| 113 | 259-280 | Leucine zipper |
| 120 | 27-40 | MAT8 family |
| 122 | 123-125 | Cell attachment sequence |